Instituto Fundación Teófilo Hernando

Departamento de Farmacología y Terapéutica

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Doctoral Thesis

Development, validation and application of mass spectrometry methods for clinical pharmacokinetics, neurotransmitter and human habitat studies

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CERTIFICAN:

Que **Doña Aneta Wojnicz**, Licenciada en Biotecnología por la Universidad Warmińsko-Mazurski en Olsztyn (Polonia), ha realizado la presente Tesis Doctoral **"Development, validation and application of mass spectrometry methods for clinical pharmacokinetics, neurotransmitter and human habitat studies"** y que a su juicio reúne plenamente todos los requisitos necesarios para optar al **Grado de Doctor**, a cuyos efectos será presentada en la Universidad Autónoma de Madrid, autorizando su presentación ante el Tribunal Calificador.

Y para que así conste se extiende el presente certificado,

Madrid, Noviembre 2016.

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V^{o}B^{o} Codirectora:

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ABSTRACT/RESUMEN

Mass spectrometry (MS) is an analytical technique, which identifies, quantifies and provides structural information about compounds in simple and complex mixtures. Herein, we have included 5 MS-based approaches. The first 4 targeted, liquid chromatography-tandem MS (LC-MS/MS) methods have been successfully validated according to the recommendations of the regulatory agencies, including tests of precision and accuracy, recovery and matrix effect, sensitivity, stability and in some cases carry-over effect. However, for the 5th untargeted metabolomics, three-dimensional (3D) surface imaging MS (3D-surface-IMS) approach, different validation strategy has been applied.

The first part of the work is focused on the drug monitoring in human plasma. The first approach enables simultaneous plasma determination of albendazole and its main metabolite albendazole sulfoxide in a fast, easy and robust manner. The second one is able to monitor omeprazole plasma levels. Both methods have been applied for pharmacokinetic studies, indicating the applicability in clinical practice. These simple assays will permit to assess adherence to treatment, therapeutic efficacy and drug resistance, improving the pharmacological treatment of patients.

The second part of this study focused on neurotransmitters' monitoring in tissue and cell cultures. First method enables the quantification of 8 neurotransmitters (monoamine and amino acids as well as some of their metabolites) in rat brain extracts. It was successfully applied in a murine depression model. The second LC-MS/MS technique is an improvement of the before-mentioned neurotransmitters approach, as it includes simultaneous determination of 14 neurotransmitters (monoamines, amino acids, nucleotides and neuropeptides) in bovine chromaffin cell cultures (BCCs). It was applied to investigate the neurosecretion profile of BCCs and also of human plasma samples, where altered neurotransmitters under stress situations such as Alzheimer's disease, are found.

Finally, the third part of this thesis describes a LC-MS and MS/MS based untargeted metabolomics method. The chemistry associated with modern human habitat was investigated and visualized with 3D-surface-IMS. This study helps to understand how chemicals are distributed on humans and their environment and may ultimately impact the design of future human habitats.

To summarize, we present 5 different MS based approaches successfully applied to different research purposes. On the whole, these results are yet another example of the great potential of a 100 years old MS technique.

Espectrometría de masas (MS, del inglés “Mass spectrometry”) es una técnica analítica, la cual identifica, cuantifica y proporciona la información sobre la estructura química de los analitos en una solución simple y compleja. En este contexto, en esta tesis doctoral, hemos incluido 5 métodos basados en la MS. Cuatro primeros métodos del análisis dirigido, basados en la cromatografía líquida de alta eficacia acoplada a la MS en tándem (LC-MS/MS), han sido validados según los requisitos de las agencias reguladoras, incluyendo los ensayos de la precisión, exactitud, recuperación de la extracción, efecto matriz, sensibilidad, estabilidad y en algunos casos del efecto arrastre. Sin embargo, para el quinto método del análisis no-dirigido y de la MS con la imagen tridimensional de superficie (3D-surface-IMS, del inglés “Three-dimensional-surface-imaging MS”), se aplicó una estrategia de validación diferente.

La primera parte de la tesis doctoral se centra en la monitorización de los fármacos en el plasma humano. El primer método facilita la determinación simultánea del albendazol y su metabolito en el plasma, de forma rápida, simple y robusta. El segundo método permite la cuantificación de los niveles plasmáticos del omeprazol. Ambas técnicas se aplicaron en los estudios farmacocinéticos, demostrando la utilidad clínica. Estos simples métodos evaluarán la adherencia, eficacia y resistencia al tratamiento, mejorando el tratamiento farmacológico de los pacientes.

La segunda parte de esta tesis trata de la determinación de los niveles endógenos de neurotransmisores en el tejido y en los cultivos celulares. El primer método permite el estudio de 8 neurotransmisores en el extracto del cerebro de rata y se aplicó con éxito en el estudio de neurotransmisores en un modelo murino de depresión. El segundo método, tras un par de modificaciones del anterior, nos permite monitorizar simultáneamente 14 neurotransmisores en las células cromafínes bovinas (BCCs, del inglés “Bovine chromaffin cells”) y sirvió para la investigación del perfil neurosecretor de las BCCs bajo condiciones de estrés, así como en la enfermedad de Alzheimer, donde los neurotransmisores también se encuentran alterados.

La tercera parte del trabajo, está centrada en el estudio metabolómico no-dirigido. 3D-surface-IMS permitió el estudio de las sustancias químicas asociadas con el entorno humano moderno con el fin del entendimiento de las interacciones entre los humanos y su entorno. Así como un diseño consciente de futuros hábitos humanos.

En resumen, presentamos 5 métodos basados en MS, aplicadas con éxito en diferentes abordajes de investigación. Los resultados incluidos en esta tesis doctoral, representan un ejemplo más del gran potencial de la MS que lleva más de 100 años.

TABLE OF CONTENT

ABBREVIATIONS AND ACRONYMS

α3-$GABA_A$ R	α3-containing $GABA_A$ receptors	DOPAC	3,4-Dihydroxyphenylacetic acid
ABZ	Albendazole	2D	Two-dimensional
ABZOX	Albendazole sulfoxide	3D	Three-dimensional
ABZO-D_3	Isotope-labeled albendazole sulfoxide	3D-surface-IMS	Three-dimensional-surface-imaging mass spectrometry
ACh	Acetylcholine	e^-	Electron
AD	Adrenaline	ED	Electrochemical detection
ADME	Adsorption, distribution, metabolism, elimination	EMA	European Medicines Agency
ADP	Adenosine 5′-diphosphate	ESI	Electrospray ionization
AMP	Adenosine 5′-monophosphate	FDA	U.S. Food and Drug Administration
AUC	Area under the curve	FLD	Fluorescence detection
APCI	Atmospheric pressure chemical ionization	GABA	γ-aminobutyric acid
ATP	Adenosine 5′-triphosphate	Glu	Glutamic acid
BCCs	Bovine chromaffin cells	GNPS	Global Natural Product Social molecular networking
Ca^{2+}	Calcium	5-HIAA	5-hydroxyindoleacetic acid
C18	Octadecyl carbon chain	5-HT	Serotonin
cAMP	Cyclic adenosine 5′-monophosphate	HILIC	Hydrophilic interaction chromatography
CAPB	Cocamidopropyl betaine	H+/K+ ATPase	Hydrogen/potassium ATPase, gastric enzyme
CE	Capillary electrophoresis	HPLC	High-performance liquid chromatography
CE-UV	Capillary electrophoresis-ultraviolet detection	HPLC-ED	High-performance liquid chromatography-electrochemical detection
C_{max}	Maximum plasma concentration	HPLC-FLD	High-performance liquid chromatography-fluorescence detection
C_{min}	Minimum plasma concentration	HPLC-NACE	High-performance liquid chromatography-non-aqueous capillary electrophoresis
CNS	Central nervous system	HPLC-UV	High-performance liquid chromatography-ultraviolet detection
CORT	Corticosterone	HIST	Histamine
CSF	Cerebrospinal fluid	H-OME	5-hydroxyomeprazole
CV	Coefficient of variation	Hybrid Q-orbitrap MS	Hybrid Quadrupole orbitrap-mass spectrometer
CYP	Cytochrome P450	HRMS	High resolution mass spectrometry
D-Ala^2-LENK	Isotope-labeled leucine enkephalin	IMS	Imaging mass spectrometry
DA	Dopamine	INY	Injection volume
DESI-IMS	Desorption electrospray ionization-imaging mass spectrometry	IR-MALDESI-MS	Infrared matrix-assisted laser desorption/electrospray ionization-mass spectrometry
DiB	Data in Brief	IS	Internal standard
dMRM	Dynamic multiple reaction monitoring	KH	Krebs Hepes buffer

KO	Knockout	Nrf2	Nuclear factor (erythroid 2-derived)-like 2
LA-IPC-MS	Laser ablation inductively coupled plasma mass spectrometry	NS	Not significant
LC	Liquid chromatography	OME	Omeprazole
LC-MS	Liquid chromatography-mass spectrometry	OME-D_3	Isotope-labeled omeprazole
LC-MS/MS	Liquid chromatography-tandem mass spectrometry	OME-S	Omeprazole sulfone
LC-HRMS	Liquid chromatography-high resolution mass spectrometry	OPFRs	Organophosphate flame retardants
LDCVs	Large dense core vesicles	PAHs	Polycyclic aromatic hydrocarbons
LENK	Leucine-enkephalin	PCoA	Principal coordinate analysis
LLE	Liquid-liquid extraction	PCR	Polymerase chain reaction
LLOQ	Lower limit of quantification	PPT	Protein precipitation
LOD	Limit of detection	PRODUCT-ION	Scanning of all product ions
m/z	Mass-to-charge ratio	Q	Quadrupole
MALDI	Matrix-assisted laser desorption/ionization	Q2	Collision cell (2^{nd} quadrupole)
MALDESI	Matrix-assisted laser desorption/electrospray ionization	QC	Quality control
ME	Matrix effect	QQQ	Triple quadrupole
MEC	Minimum effective concentration	R	Recovery
$[M+H]^+$	Charged ion by the addition of a hydrogen cation	R^2	Correlation coefficient
MENK	Methionine-enkephalin	RIA	Radioimmunoassay
MHPG	3-Methoxy-4 hydroxyphenylglycol	RT	Retention time
MN	Metanephrine	SCAN	Scanning of all ions
MRM	Multiple reaction monitoring	SIM	Selected Ion Monitoring
MS	Mass spectrometry	SD	Standard desviation
MS1 (=Q1)	1^{st} mass analyser (1^{st} quadrupole)	SIMS	Secondary ion-mass spectrometry
MS2 (=Q3)	2^{nd} mass analyser (3^{rd} quadrupole)	SPE	Solid phase extraction
MTC	Maximum tolerated concentration	TDM	Therapeutic drug monitoring
Mw	Molecular weight	t_{max}	Time to reach maximum concentration
NACE	Non-aqueous capillary electrophoresis	TOF	Time-of-flight detector
nanoDESI-MS	Nanospray desorption electrospray ionization-mass spectrometry	TOF-SIMS	Time-of-flight-secondary ion-mass spectrometry
NI	Not informed	UHPLC (=UPLC)	Ultra high-performance liquid chromatography
NTs	Neurotransmitters	UV	Ultraviolet detection
NTT	No treatment	WT	Wild-type

INTRODUCTION

Introduction

1. Importance and scope of LC-MS in all fields of analytical chemistry

Liquid chromatography-mass spectrometry (LC-MS) was originally developed in the field of analytical chemistry. Although LC and MS are widely used separately, the coupling of LC to MS as a hyphenated method showed a great advantage in comparison to the other methods (Nikolin et al., 2004). LC-MS offered unmatched sensitivity, detection limits, speed and diversity of applications, and for this reason it has rapidly become one of the most important analytical techniques (de Hoffmann and Stroobant, 2007; Griffiths, 2008; Skoog et al., 2014). Hundred years old MS, invented by J. J. Thomson in 1913 (Thomson, 1913), enables identifying of truly unknown compounds, quantifying of known compounds and exploring molecular structures (Maher et al., 2015). This wonderful approach is widely applied in almost any area of research. It became very important in the last 10 years as it has been successfully applied in forensic sciences (Skoog et al., 2014), clinical toxicology (Wu et al., 2012a), pharmaceutical analysis (Nikolin et al., 2004) and laboratory medicine (Wallemacq, 2011). Even ion analysis in space (Petrie and Bohme, 2007) and Mars exploration (Hoffman et al., 2008) were performed using MS. Thus, LC-MS was named by Bowers as the state-of-art for the drug analysis laboratory (Bowers, 1989).

Therefore, given the sensitivity and the robustness of this technique, we used it for the following purposes:

- clinical pharmacokinetic studies
- neurotransmitters research
- modern human habitat studies.

In this work, 5 publications and one Data in Brief (DiB) related to the 3rd article are included. The first four publications and the DiB describe 4 developed and validated targeted MS methods, with their application for clinical pharmacokinetic studies (Wojnicz et al., 2013; Wojnicz et al., 2015) and neurotransmitters research (Wojnicz et al., 2016a; Wojnicz et al., 2016b; Wojnicz et al., 2016c). The 5th publication reports an example of untargeted metabolomic method using IMS in modern human habitat studies (Petras et al., 2016).

2. General concepts

2.1. Principles of LC

Chromatography has been developed by the Russian botanist Tswett in the early 20th century (Fornstedt et al., 2015; Strain and Sherma, 1967), when he applied paper chromatography for plant chlorophyll separation. Chromatography is an analytical technique based on the separation of compounds of a mixture due to differences in their structure and/or composition. As a general concept, chromatography involves moving a sample via mobile phase through the system over a stationary phase. Since the first application, many types of chromatography have appeared, although gas chromatography and LC are the most preferred in laboratory practice (Snyder et al., 2011). Of note, LC has become more important than gas chromatography and it is being used in practically all chemistry fields.

In LC, the mobile phase is a mixture of solvents; while the stationary phase is an analytical column. The separation of the molecules occurs depending on specific affinities and interactions with the stationary support. Analytes that interact more strongly with the stationary phase will move more slowly through the column than components with weaker interactions. Different compounds will move with different velocity through the column, thus they can be separated from each other. High-performance liquid chromatography (HPLC), often also called LC for simplification, is a type of LC used to separate, qualify and quantify compounds under high pressure (up to 400 bar) that have been dissolved in a solution (Kupiec, 2004; Rossomando, 2006; Weston and Brown, 1997a). Other improvement of HPLC is the ultra HPLC (called UHPLC or UPLC), which is designed to work under extremely high pressure (up to 600 or even 1000 bar), offering better analyte separation and better sensitivity (Fornstedt et al., 2015).

HPLC components and workflow

The components of the common used HPLC system are:

(1) Solvents (mobile phase)

(2) High pressure pump

(3) Sample injector (autosampler)

(4) Analytical column (stationary phase)

(5) Detector (instrument control and data acquisition).

Figure 1 shows an example of a typical HPLC system used in analytical laboratories. Briefly: (1) a reservoir that holds the solvent, mobile phase; (2) a high-pressure pump used to generate and apply a specified flow rate of mobile phase, typically mL/min. The high-pressure pump can be binary (it can pool two different solvents at the time) or quaternary (four different solvents); (3) autosampler injects the sample into the HPLC column; (4) the column contains the chromatographic packing material for analyte separation in time; (5) a detector transforms and amplify the signal of the analytes that elute from the HPLC column; and (6) a computer performes data acquisition and chromatograms generation (Rossomando, 2006; Weston and Brown, 1997b).

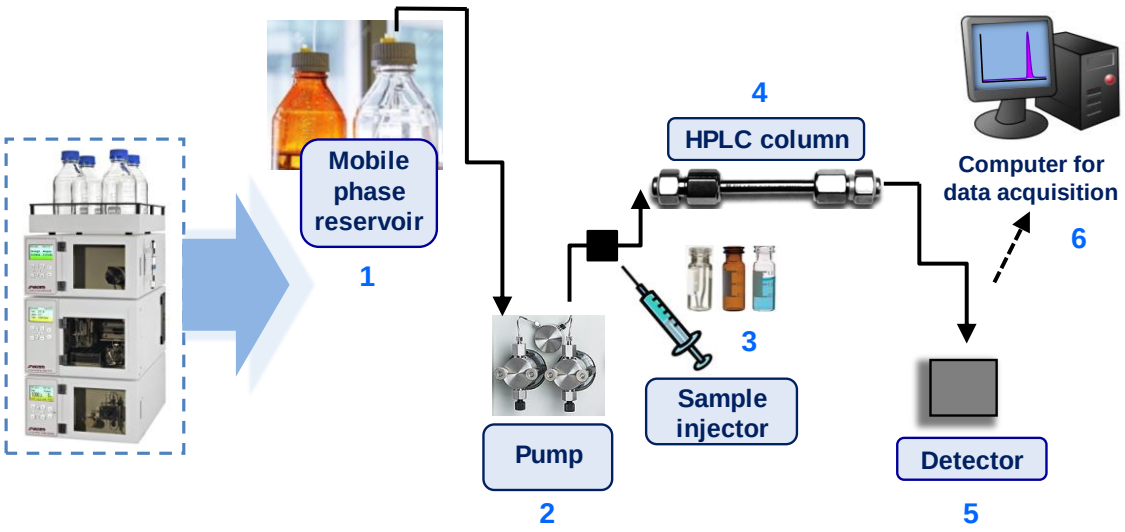

Figure 1. Components and simplified workflow of HPLC system. Abbreviation: HPLC: high-performance liquid chromatography. Modified from www.agilent.com and www.waters.com

Retention time

The time that an analyte is retained on an analytical column by the stationary phase is defined as the retention time (RT). This is measured from the time of sample injection to the time of analyte elution; thus a chromatogram is generated (Snyder et al., 2011). **Figure 2** demonstrates a typical representative chromatogram of albendazole (ABZ), a drug analysed in our Analytical and Pharmacokinetic Unit, Department of Clinical Pharmacology, Hospital Universitario de la Princesa, Madrid. With a given column and solvent, RT is characteristic for analyte and provides qualitative information, while area or height of the peak permits to obtain quantitative information (Fornstedt et al., 2015; Skoog et al., 2014). Thus, to determine the concentration of an analyte, the peak area or height of calibration standard with known concentration is plotted against the peak area or height of the analyte.

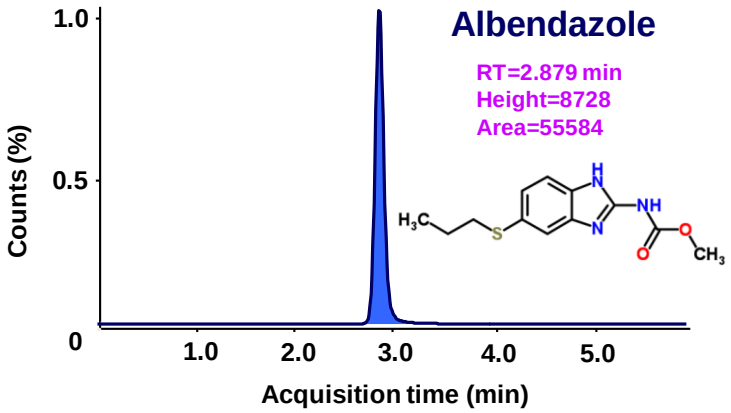

Figure 2. Representative chromatogram of albendazole. Counts (%) versus acquisition time (min). Abbreviation: RT: retention time. Reproduced and adapted from Wojnicz et al., 2013.

Analytical column and mobile phase

As mentioned earlier, RT of the analyte depends mainly on the mobile phase and the analytical column applied. The term “normal” chromatography comes from the use of a stationary phase or column, which contains unmodified silica or alumina resins with polar or hydrophilic properties. On the contrary, the use of non-polar or hydrophobic column is the opposite of “normal-phase”; hence the name of “reversed-phase” chromatography. In this case, analytical column is usually packed with porous silica gel or polymer particles with chemical ligands bound on their surface. The most common material is the octadecyl carbon chain (C18)-bonded silica. On top of that, different solvents are used for different types of chromatography. For “normal-phase” chromatography, the solvent is usually non-polar (hexane or heptanes); in “reverse-phase” chromatography, the solvent is normally a mixture of water and a polar organic solvent (acetonitrile or methanol). The purity of all solvents used for the analysis should be of HPLC grade.

Choosing the correct column is very important. Polar molecules, for example, are poorly retained on “reverse-phase” columns and elute at the beginning of the chromatogram with a low organic content. One way to overcome this problem is to use hydrophilic interaction chromatography (HILIC), in which polar analytes are retained and eluted with higher organic content of the mobile phase (Pitt, 2009). Analytical columns have a pH range usually between 2 and 9 (specific for each column), that should be taken into account when the pH of mobile phase is optimized. Temperature of the column can be set in order to optimize separation of the analytes. Other important aspect to take into account is the column resolution, defined as the ability of the column to separate peaks on the chromatograph (Kupiec, 2004). Especially when

the separation of many different compounds is performed, the resolution of the column will play an important role. Column care should be considered in the daily performance. Washing methods should be designed for column clean up and strong basic or acidic solvents, such as untreated samples, must be avoided.

Volume of sample injection

Sample injection volumes for ordinary packed analytical columns vary from a few tenths of a microliter to 20 µL (Skoog et al., 2014). In detail, sample injection volume depends on the length of the column, particle size and detector applied. For example, for a conventional HPLC column with the particle size of 3-5 µm, optimal injection volume will be around 5-10 µL, while for a UPLC column with the particle size <2 µm, the injection volume should not exceed 1-2 µL.

Chromatographic separation

The chromatographic separation can be performed in an isocratic or gradient elution. In the isocratic method, the composition of the solvent or solvent mixture (mobile phase) is constant during the separation process, while in the gradient method the composition of the mobile phase (mixture of polar and apolar solvents) changes with time (Scott, 1992; Snyder et al., 2011). Isocratic elution is recommended as the 1st method of choice, especially when analytes of interest are easily separated isocratically, or when the sample contains less than 10 weakly retained compounds and the gradient baseline impedes trace analysis. However, it has been shown that gradient elution can provide a faster analysis, narrower peaks and similar resolution compared with isocratic separation (Schellinger and Carr, 2006). Another aspect to optimize when an analytical method is being developed is the flow rate applied for chromatographic separation. It depends on the type of analytical column and the pressure limits of chromatographic system (Fornstedt et al., 2015).

Detectors and hyphenated techniques

LC can use many different types of detectors, and some of them have so far found limited application in gas chromatography (Snyder et al., 2011). Nevertheless, detectors relevant to this work are listed below:

- electrochemical detector (ED)
- ultraviolet (UV)
- fluorescence detectors (FLD)

- MS detector.

Each detector has its assets, limitations and sample types for which it is most effective. Many clinical analytical labolatories apply HPLC coupled to UV (HPLC-UV) for drug analysis (Kupiec, 2004). However, nowadays MS detectors are becoming more and more popular due to their compounds structure identification and the ability to quantify the analytes at very low detection limits (LOD). Within this context, the coupling of MS to chromatographic techniques has always been desirable due to the sensitive and highly specific nature of MS compared to other chromatographic detectors (Pitt, 2009). Separation techniques, such as liquid and gas chromatography or capillary electrophoresis (CE) coupled to MS, infrared spectroscopy or nuclear magnetic resonance named hyphenated techniques, are nowadays widely used in analytical laboratories. The 1st hybrid method, LC coupled to MS or to tandem MS (MS/MS) was developed in the last century (Baldwin, 1973; Tal'roze, 1968), providing significant improvement of sensitivity and selectivity for complex liquid mixtures.

In the present work, LC coupled to MS/MS (LC-MS/MS), such as UPLC coupled to hybrid Quadrupole-orbitrap MS (hybrid Q-orbitrap MS), was used for method development and validation due to its sensitivity, selectivity, accuracy, precision, flexibility, robustness, versatility and speed. Thus, a detailed introduction to MS will be provided next.

2.2. Principles of MS

A bit of history

MS was developed within the field of physics. The first MS was invented by Nobel laureate Sir J. J. Thomson in 1913 (Thomson, 1913). He described "parabola spectrograph" in his monograph entitled "Rays of Positive Electricity and Their Application to Chemical Analysis". This "parabola spectrograph" was able to measure ion intensity and produce a mass spectrum. Originally, the technique was used to measure masses of atoms and has served to demonstrate the existence of isotopes of the elements. By the 1940s, the principal application of this technique was in the petroleum industry (Griffiths, 2008). Nowadays, it has an application in almost any field of science.

How MS works: Components and workflow of MS

MS generates multiple ions from the sample, according to the mass-to-charge ratio (*m/z*) of constituent molecules. The "*m"* refers to the molecular or atomic mass number, while the "*z"* to the charge number of the ion (Skoog et al., 2014) and it is specific for each substance. This rationale allows qualitative and quantitative determination of compounds as well as obtaining structural information.

Many instruments exist and vary widely in size, resolution, flexibility and cost; however, their components are remarkably similar. The workflow of mass spectrometric analysis consists of 3 steps: ionization of the sample, separation according to their *m/z* and detection. Moreover, the instrument needs sample inlet and data output (computer) to work correctly. Thus, a typical mass spectrometer consists of the following 5 components:

(1) Sample introduction site (solid samples, liquids and gases may be introduced)

(2) Ion source (uncharged compounds of the sample are converted into ions)

(3) Mass analyser (separation of ions according to their *m/z* ratios)

(4) Detector

(5) Data output site (instrument control and data acquisition).

Figure 3 ilustrates an example of a typical mass spectrometer and its simpliefied workflow.

(1) Sample introduction site: Sample can be introduced directly to the MS system; however, usually it is previously separated by LC before MS analysis.

(2) Ion source. After sample introduction, analytes are ionized using an ion source. The uncharged analyte is converted into posite or negative charged ions. John Fenn (Fenn, 2003) and Koichi Tanaka (Tanaka, 2003) received Nobel Prize in chemistry for their development of "soft desorption ionization methods for mass spectrometric analyses of biological macromolecules". Electrospray ionization (ESI), by John Fenn (Fenn et al., 1989), and matrix-assisted laser desorption/ionization (MALDI), by Koichi Tanaka (Tanaka et al., 1988), enable successful ionization of large fragile biomolecules. Another ion source is atmospheric pressure chemical ionization (APCI). Of these, ESI is one of the most popular nowadays, and it works by applying high voltage to the liquid sample and thus producing charged liquid droplets.

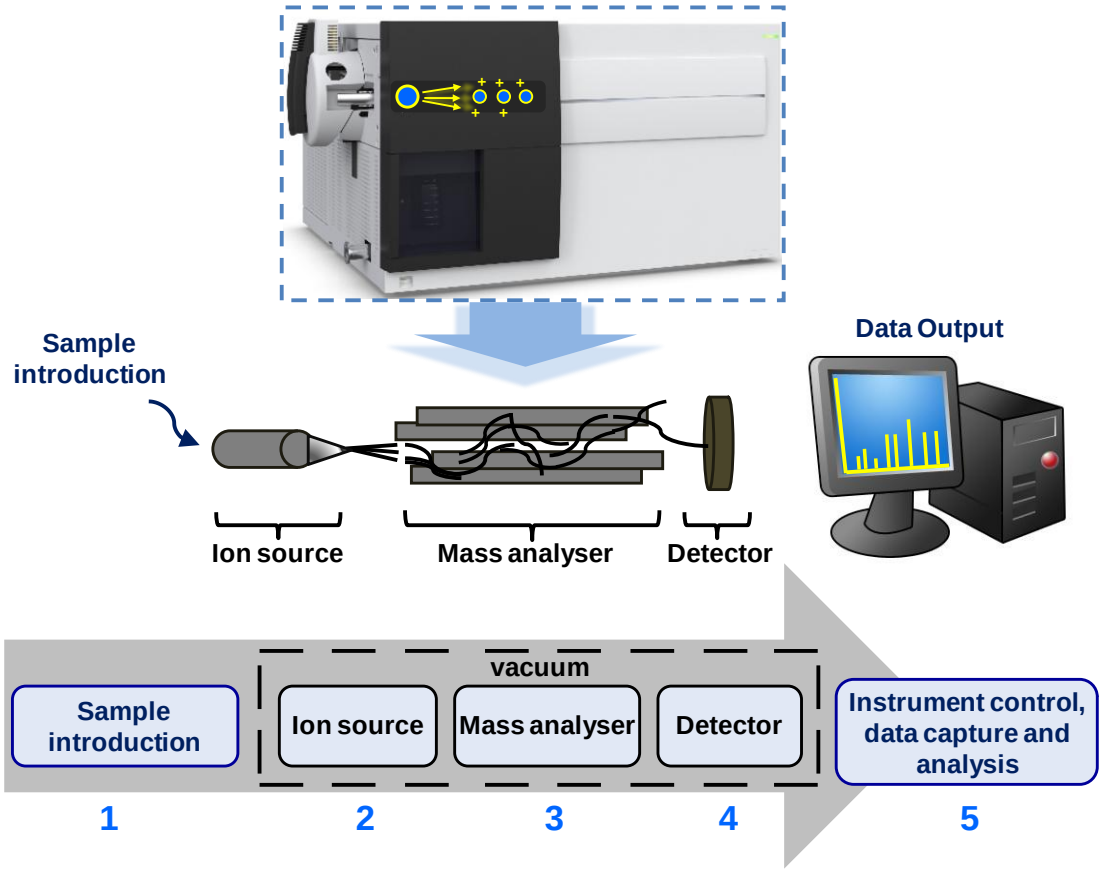

Figure 3. Components and workflow of mass spectrometer. Reproduced and modified from Maher et al., 2015.

(3) Mass analysers. All ions are separated in the MS according to their *m/z*. The separation and (typically) the ionization processes are carried out in vacuum (Maher et al., 2015; Skoog et al., 2014). **Figure 3** displays a typical mass spectrometer with one mass analyser (MS). The most common mass analysers are: time-of-flight (TOF), orbitraps, quadrupoles (Q) and ion traps. Each mass analyser has different characteristics and therefore different applications. Mass s can be used to separate all analytes in a sample for targeted and/or untargeted screening.

(4) Detection: Finally, the ions are detected in proportion to their abundance (signal intensity). After ionization and separation, the produced ions pass to the transducer, which converts the number of ions (abundace) into an electrical signal as a meaningful output for the user. There are several types of ion traducers available, but the most common is the electron multiplier. Fourier transform is another detector type, also used in some instruments.

(5) Data output site: Usually, a computer performes the instrument control and data acquisition.

After MS analysis, mass spectrum of the molecule is produced. It provides results as a plot of ion abundance (%) versus *m/z* (de Hoffmann and Stroobant, 2007). **Figure 4** shows an example mass spectrum of omeprazole (OME), recording a prodrug very often used for stomach ulcer treatment.

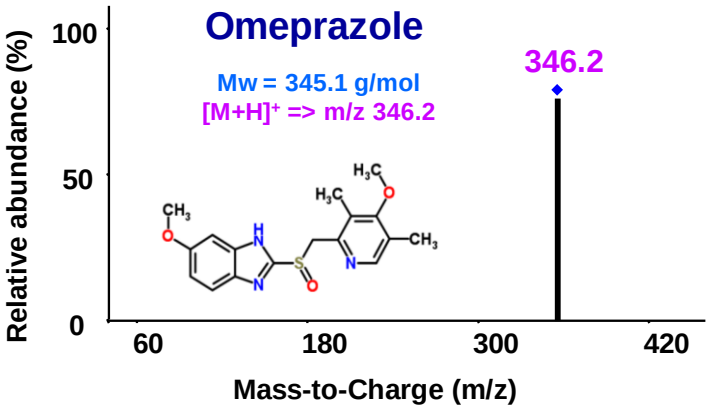

Figure 4. Mass spectrum of omeprazole. Relative abundance (%) versus *m/z*. Abbreviations: Mw: molecular weight; *m/z:* mass-to-charge ratio; $[M+H]^+$: charged ion by the addition of a hydrogen cation. Reproduced and adapted from Wojnicz et al., 2015.

In this work, 2 types of MS have been used:

- MS/MS: it was used for first 4 methods' development and validation,
- hybrid Q-orbitrap MS: this type of instrument was used for the 5th method.

Therefore, I will describe in depht their components and how they work.

MS/MS and modes of work

MS/MS, called also triple quadrupole (QQQ), consists of 2 analysers connected in space (MS/MS), with a collision cell between them. Hence, it's name QQQ. The 1st actual physical arrangement of MS/MS was designed by Lindholm in 1954 (Lindholm, 1954). **Figure 5** shows the components of a MS/MS instrument that consist of 3 parts:

(1) Ion source (ESI, APCI)

(2) Mass analyser (QQQ):

(2a) Q1: The 1st mass analyser, called also MS1. It is the filter for precursor ions

(2b) Q2: The collision cell. Here, the precursor ions undergoe fragmentation and produce product ions

(2c) Q3: The 2nd mass analyser, called also MS2. This part analyses and filters product ions from collision cell

(3) Detector

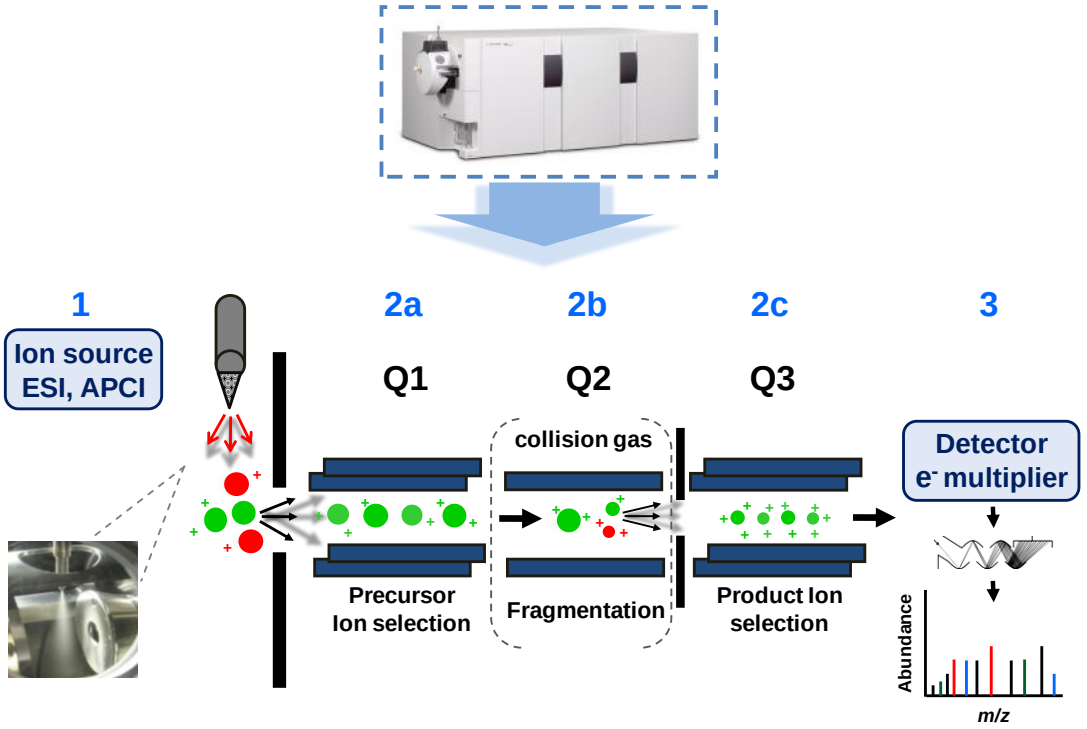

Figure 5. Components of MS/MS instrument. Ionization source, followed by the 1st mass analyser (Q1), the collision cell (Q2) and the 2nd mass analyser (Q3). Finally the detector, usually electron multiplier, is included. Abbreviations: APCI: atmospheric pressure chemical ionization; e-: electron; ESI: electrospray ionization; m/z: mass-to-charge ratio. Modified from Maher et al., 2015.

Briefly, the ions pass from HPLC to the ion source (1) that can be either APCI or ESI source. In the next step, generated ions pass through mass analysers (QQQ). Each of the 2 mass filters (Q1 and Q3) contains four parallel, cylindrical metal rods while the collision cell, Q2, is filled with ultrapure collision gas (N_2 or Argon). In the 1st mass analyser (Q1), the precursor ions are filtered (2a). Subsequently, in the collision cell (Q2), precursor ions undergoe fragmentation and the fragments (product) ions are generated (2b). Produced fragments are then filtered by the 2nd mass analyser (Q3; 2c) and conducted to the detector (3), which is generally the electron multiplier.

This type of instrument offers additional advantage for targeted analysis, since it gives information not only about specific precursor ion, but also about product ions from collision cell by collision-induced dissociation (Maher et al., 2015).

In MS/MS instrument, 4 different modes of work, depicted in **Figure 6,** exist:

- Mode 1: MS2-SCAN performs scanning of all precursor ions, which are entering the 2nd mass analyser (Q3). No ion fragmentation occurs; all precursor ions enter MS2 and are plotted into the mass spectrum.

- Mode 2: MS2-SIM carries out scanning of selected precursor ion(s). In this mode, we need to optimize one of the most relevant parameters in MS/MS, the declustering energy, which accelerates the ions outside the dielectric capillary in order to favor the conductance of only selected precursor ion(s) to the 1st Q.

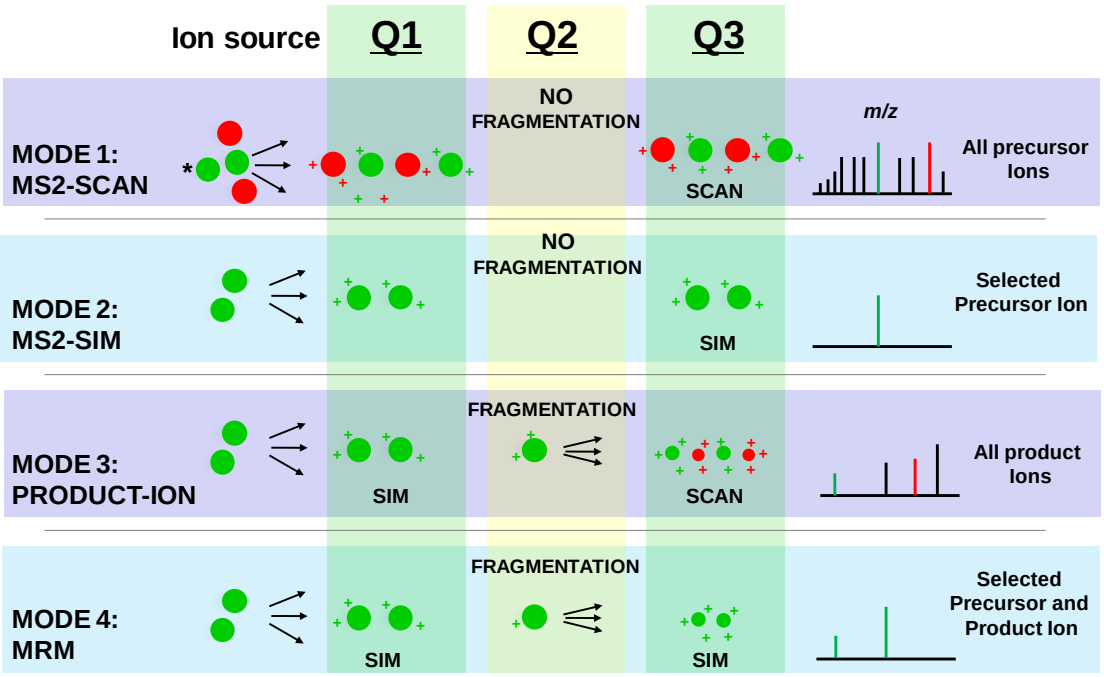

Figure 6. Modes of work of MS/MS. Abbreviations: MS/MS: tandem mass spectrometry; SCAN: scanning of all ions; SIM: selected ion monitoring. MS2-Scan: scanning of all precursor ions, MS2-SIM: scanning of selected precursor ion, PRODUCT-ION: scanning of all product ions from Q2; MRM: Multiple Reaction Monitoring, performs scanning of selected precursor ion(s) and selected product ion(s). Modified from Maher et al., 2015.

- Mode 3: PRODUCT-ION performs scanning of all product ions that are produced in collision cell (Q2) from a selected precursor ion. In this mode, the collision energy, which fragments the precursor ion into the product ions, is also optimized.

- Mode 4: In Multiple Reaction Monitoring (MRM), the instrument performs the monitoring of selected precursor ion(s) and selected product ion(s), which enables monitoring of not only one ion transition, but also 2 or more ion *m/z*, even with very similar RT. In this mode, declustering and collision energy parameters are finally checked and optimized.

QQQ is characterized by limited resolution (about 1,000). Resolution typically is expressed as a unitless number that is the ratio of peak *m/z* ratio divided by the peak

width at half its height (full width/half maximum). QQQ resolution is acceptable for the quantification of known analytes, but it is often not sufficient for the screening of unknown compounds.

Hybrid Q-orbitrap MS

In the 5th publication included in the present thesis, a hybrid instrument, consisting of a Q mass filter and an orbital ion trap mass filter (orbitrap), was used. The orbitrap is an ion trap mass analyser that employs trapping of ions in an electrostatic field (Hu et al., 2005; Makarov, 2000). Hybrid Q-orbitrap MS used for the 5th publication consists of the following parts:

(1) Ion source (APCI or ESI)

(2) Mass analyser:

(2a) Quadruple (1st mass analyser, MS1)

(2b) C-trap

(2c) Collision cell

(2d) Orbitrap (2nd mass analyser, MS2)

(3) Detector (Fourier transform)

Figure 7 shows the components and simplified workflow of hybrid Q-orbitrap MS.

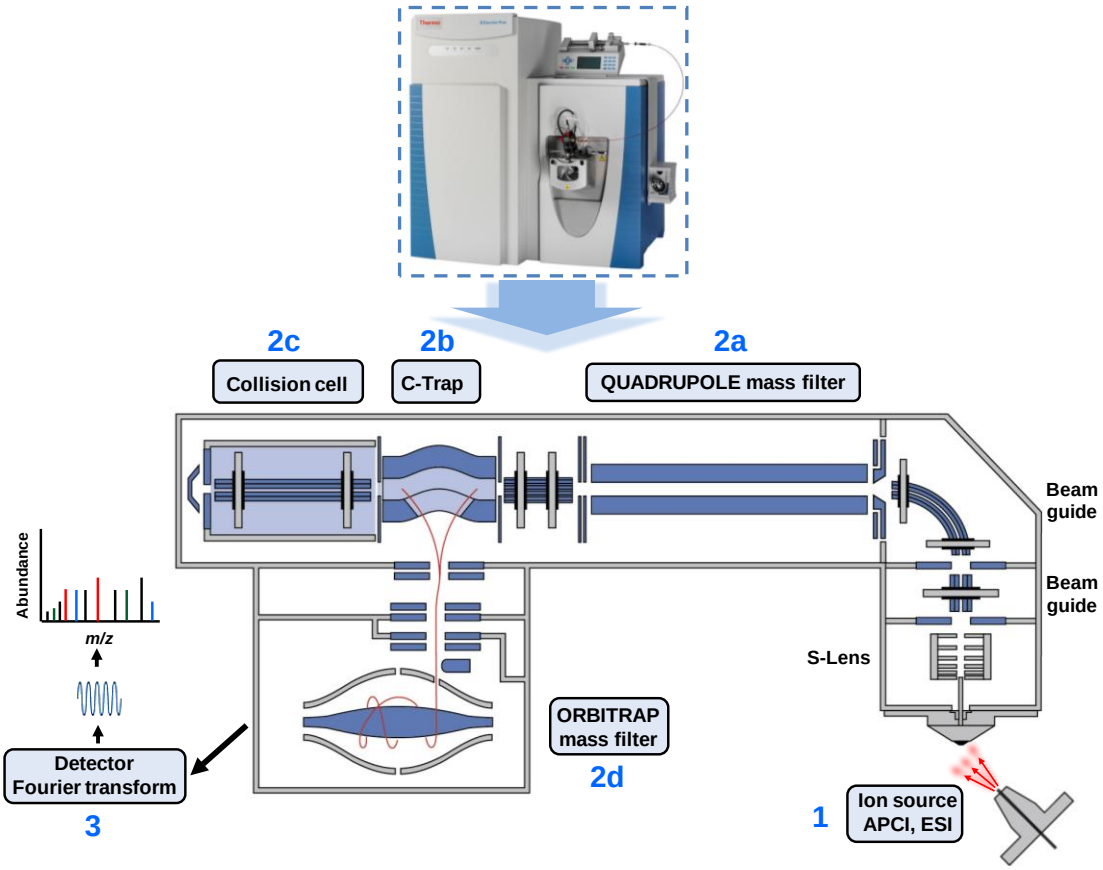

Figure 7. Scheme of the hybrid Q-orbitrap MS. It includes an ion source (1) followed by quadrupole mass filter (2a), next by "C-trap" (2b) and collision cell (2c). "C-trap" allows storage of a bundle of ions and radial ejection toward the orbitrap mass filter (2d). Ions are detected using the Fourier transform detector (3). Abbreviations: APCI: atmospheric pressure chemical ionization; ESI: electrospray ionization; hybrid Q-orbitrap MS: hybrid quadrupole-orbitrap mass spectrometer. Modified and adapted from www.thermo.com and Macarov, 2000.

Briefly, the ions pass from UPLC to the ion source (1), in this case APCI or ESI. The ions are captured and focused for effective ion transmission using S-lens and pass next to the active beam guide, where uncharged neutral species are filtered out. In the next step, generated ions are filtered in the 1st mass analyser, the Q (2a) and then ejected axially into the C-trap (2b). The C-trap is used to store and slow down the ions before injecting them into the Collision cell (2c) or to the 2nd mass analyser, the orbitrap (2d). When precursor ions pass directly from C-trap to the orbitrap mass analyser, MS analysis is performed. However, when precursor ions pass through collision cell, before injection to the orbitrap mass analyser, product ions are produced and then MS/MS analysis is performed. Next, the ions transferred from the C-Trap are captured in the orbitrap by a rapid increase in the electric field. The signal current from the trapped ions is detected (3) and converted to a mass spectrum using the Fourier transform of the frequency signal.

In hybrid Q-orbitrap MS, high-mass resolution (up to 140,000) along with high-energy acceptance and wide mass range have been demonstrated (Makarov, 2000). S-Lens is applied for increased sensitivity, thus this type of instrument is highly recommended for untargeted screening in proteomic and metabolomic studies.

The principles of LC and MS have been explained in points 2.1 and 2.2. Another important issue before LC-MS analysis is the means of sample preparation. Since it is a very critical step before injection into the LC, the following section will describe general concepts of techniques used for sample preparation, including their advantages and disadvantages.

2.3. Sample preparation for LC-MS analysis

Direct injection of the sample to the LC-MS is possible; however, when analytes of interest are present in complex matrices (tissue, blood, plasma, urine, cerebrospinal fluid [CSF]), the sample is usually not suitable for direct introduction into the analytical instrument. In addition, biological matrices have often numerous endogenous and exogenous components, such as salts, acids, bases, proteins, polar organic acids, cells, lipids and lipoproteins that can interfere with LC-MS analysis, especially when ESI is applied for sample ionization. Therefore, an appropriate and reproducible sample treatment becomes an important step in the analytical process. Sample preparation is the most critical step with the following goals: selective analyte isolation from the complex matrix; elimination or minimization of matrix effect; and, when possible, analyte concentration. Analyte recovery and matrix effect are 2 tests that should be taken into account when the sample preparation method for MS analysis is being developed. Recovery is defined as the extraction efficiency of an analytical process, reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method. Matrix effect is defined as the direct or indirect alteration or interference in response to the presence of unintended analytes (for analysis) or other interfering substances in the sample (FDA, 2001). Matrix effect test for LC with other detectors (UV, ED, FLD) is not required by the regulatory agencies.

Although simple dilution or filtration is still used in some cases, sample preparation should be understood as an additional method to optimize. Many sample preparation methods exist. However, the most common for LC-MS sample pretreatment are:

(1) Protein precipitation (PPT)

(2) Liquid-liquid extraction (LLE)

(3) Solid phase extraction (SPE).

(1) PPT involves denaturation (loss of tertiary and secondary structures) of proteins present in biological matrix by a strong acid/base/heat or by the use of an organic solvent such as acetonitrile/methanol (Englard and Seifter, 1990). (2) LLE or solvent extraction is a separation process, which is based on the different distribution of the analytes to be separated between two liquid phases (usually one is aqueous and the other one organic). An extraction can be accomplished if the analyte has favorable solubility in the organic solvent (Mitra and Brukh, 2003). (3) SPE is based on the physical-chemical processes of sorption. SPE is an extraction technique used for concentration and isolation of target analyte from complex samples. This technique employs adsorbents in cartridge, disk, or membrane format to extract (separate) different analytes from a sample (Mitra and Brukh, 2003; Pawliszyn, 2014). Sample preparation method will have direct influence on the quality of the resulting chromatogram and process of sample ionization in MS (Bourgogne and Wagner, 2015; Smith, 2003), thus on analyte recovery and matrix effect.

Table 1 displays the comparison of different sample preparation techniques that should be taken into account, when a LC-MS method is developed.

	Less selective → SAMPLE PREPARATION → Highly selective		
INTERFERENCE REMOVED	**PPT**	**LLE**	**SPE**
Particles	+	-	+
Polar organic acids	-	+	+
Proteins	+	+/-	+
Lipids	-	-	+
Oligomeric surfactants	-	-	+
Pigments	-	-	+
Salts	-	+	+

Table 1. Comparison of different sample preparation techniques. Abbreviations: PPT: protein precipitation; LLE: liquid-liquid extraction; SPE: solid phase extraction; "+": interference totally removed; "+/-": interference removed partially; "-": interference not removed. Modified from www.agilent.com, www.waters.com and www.sepscience.com.

Simple filtration can be applied as the sample preparation when any insoluble material would block the analytical column or any connexion of HPLC system. Dilution technique should be avoided for LC-MS sample preparation, because it does not remove particles, proteins, polar organic acids, lipids, oligomeric surfactants, pigments or salts. However, it can be applied in some cases, when combined with additional sample centrifugation with precipitation solution. Although PPT method eliminates

particles and protein interferences, lipids and salts are still present in the extracted sample. Similar results can be expected with LLE. Lipids, especially phospholipids, are important LC-MS contaminants, especially when ESI is applied as the ion source (Annesley, 2003; Jessome and Volmer, 2006; King et al., 2000; Matuszewski et al., 2003; Pitt, 2009). Thus, good sample preparation with successful interferences removal is recommended. SPE eliminates all interferences present in complex matrices, leading to the minimalization of the matrix effect (Shen et al., 2005).

There is no sample preparation technique that provides total elimination of matrix effect. However, SPE or LLE (because of salts elimination) reduce ion suppression, whereas simple protocols such as PPT are often associated with more matrix effect during the ionization process (Jessome and Volmer, 2006; Vogeser and Seger, 2010) and also instrument contamination. Therefore, removing interferences from the sample with a proper clean up method gives more accurate and reproducible results.

In order to develop the LC-MS method, we are going to describe 2 types of analyte screening, followed by regulatory agencies requirements needed for the validation of the LC-MS or LC-MS/MS bioanalytical method.

2.4. Targeted and untargeted screening

As highlighted before, the use of MS can be applied not only for qualitative analysis (yes/no) and quantitative analysis of known analytes, but also for qualitative analysis of unknown compounds. Therefore, the screening technique can be targeted and untargeted.

Targeted screening

Targeted analysis consists of designing an acquisition method to determine a list of known compounds, very often with the use of reference standards and internal standards (ISs), and requiring a method validation prior to analysis of real samples (Hird et al., 2014; Patti et al., 2013). There are numerous guidances for bioanalytical method validation (EMA, 2011; FDA, 2001; ICH, 1994). The advantage of this analysis is not only analyte identification, but also the measurement of its concentration in the sample. However, targeted analysis is limited to the list of available standard reference materials. Targeted approach has been widely applied for: pharmacokinetic studies (Wojnicz et al., 2013; Wojnicz et al., 2015) or drug monitoring for treatment improvement (Moreno et al., 2013); environmental pesticides (Hernandez et al., 2008) or insecticides (Mezcua et al., 2008) monitoring; endogenous neurotransmitters and

their metabolite determination with good sensitivity (Buescher et al., 2010; Wojnicz et al., 2016a; Wojnicz et al., 2016b).

Untargeted screening

Untargeted screening aims to simultaneously measure as many compounds as possible from a biological sample without any bias (Patti et al., 2013). This approach gives the possibility of detecting both unexpected compounds and true unknowns in the sample (Hird et al., 2014), and often it can maintain sufficient sensitivity for quantification. Untargeted screening produces enormous datasets in the order of gigabytes per sample, so it requires a specific software inspection. However, untargeted approach provides greater scope than targeted analysis, enabling the discovery of unknown compounds and their metabolites. Untargeted analysis is irreplaceable in proteomics and metabolomics studies. At the moment, there is no available guidance for untargeted method validation, but field specialists are intensively working on it. There are numerous examples of untargeted approach applied for: drug screening (Wu and Colby, 2016); changes of uncharacterized metabolites as a biomarker of health and disease (Baker, 2011); and more recently novel strategies for isotope-assisted metabolomics (Nakayama et al., 2014).

Figure 8 shows simplified workflow of a targeted and untargeted screening of analytes using LC-MS.

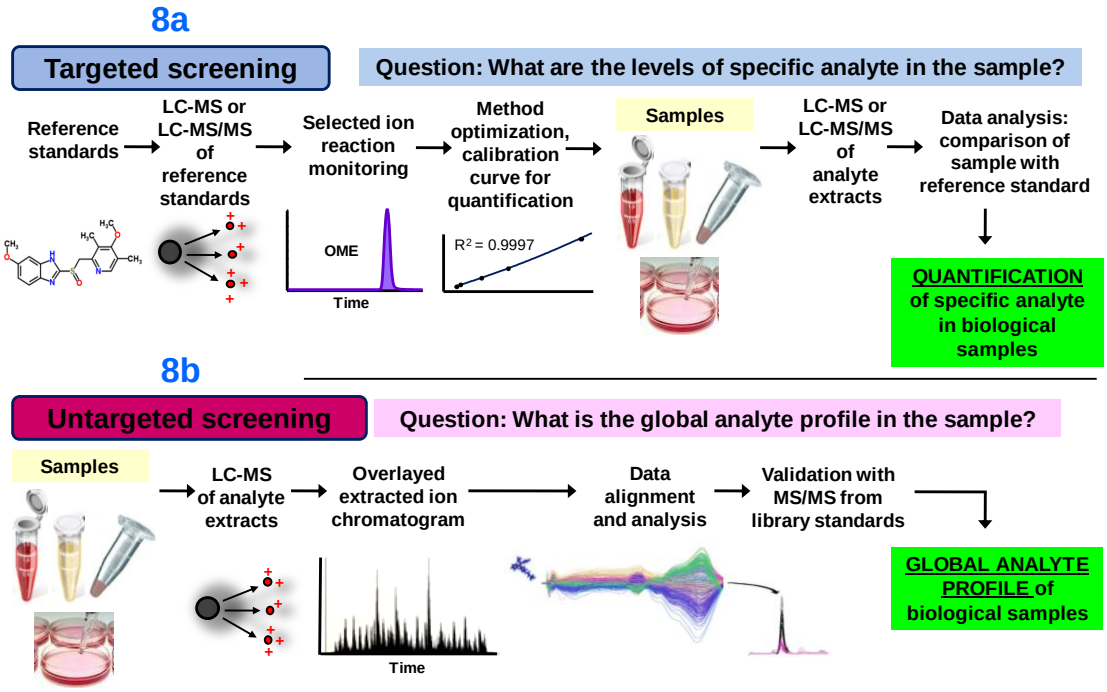

Figure 8. Workflow of targeted and untargeted screening of analytes by LC-MS. In targeted screening (8a), the optimization of LC-MS conditions for analyte of interest is the first step and then real samples are analysed after method development; while in untargeted screening (8b), first the real sample is injected into the LC-MS system and after that, the global analytes are identified using the libraries. Abbreviations: LC-MS: liquid chromatography-mass spectrometry; LC-MS/MS: liquid chromatography-tandem mass spectrometry; OME: omeprazole; R^2: correlation coefficient. Reproduced, modified and adapted from Patti et al., 2013.

2.5. Bioanalytical method validation

Validation of an analytical method includes an extensive list of procedures recommended by regulatory agencies to demonstrate that a particular method, for a particular matrix (tissue, blood, serum, plasma, urine or saliva), is reliable and reproducible for quantitative measurements of analytes.

Guidelines of regulatory agencies include the general requirements for targeted analytical method validation. However, the strategies for untargeted method validation are still under development.

Targeted method validation

Targeted method development and validation is much easier. The analyst must define the list of known compounds, often from reference standards, and follow the steps defined in validation guidelines. Regulatory agencies, such as U.S. Food and Drug Administration (FDA) (FDA, 2001) and European Medicines Agency (EMA) (EMA, 2011), established guidance's for bioanalytical method validation. Both guidances contain detailed description of validation steps required for targeted method

development, with some differences. In summary, the full validation of a bioanalytical method should meet the following requirements:

(1) Accuracy and precision

(2) Selectivity

(3) Lower limit of quantification (LLOQ) and calibration curve

(4) Extraction recovery and matrix effect

(5) Stability.

(1) Accuracy and precision: accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

(2) Selectivity: called also specificity, is the ability to assess unequivocally the analyte in the presence of components, which may be expected to be present. Typically, these might include impurities, degradants or matrix components.

(3) LLOQ and calibration curve: The LLOQ of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy (FDA, 2001; ICH, 1994). It gives the information about the method sensibility. Calibration curve is defined as the relationship between the experimental response value and the analytical concentration. Calibration curve should be performed for each analysis in order to quantify the concentration of the analyte. Additionally, quality controls (QCs) at LLOQ, low, medium and high concentration should be applied (EMA, 2011; FDA, 2001). Three different calibration methods, each with its own benefits and limitations, can be utilized in quantitative analysis: external standard, the IS and the standard addition method. We have used the IS method in validation process. In this method, an equal amount of an IS, a component that is not present in the sample, is added to both the sample and standard solutions (Kupiec, 2004). The IS selected should be chemically similar to analyte of interest and have similar RT. Ideally, when possible, IS should be isotope-labeled. IS method tends to be the most accurate and precise (EMA, 2011; FDA, 2001). Thus, a calibration curve should consist of a blank sample (matrix sample without IS), a zero sample (matrix sample with IS) and 6 to 8 calibration standards covering the expected calibration range.

(4) Extraction recovery and matrix effect: the recovery is calculated as the ratio of the peak area of the analyte added to the biological matrix before extraction to the peak area of analyte added to the biological matrix after the extraction process. Matrix effect is calculated as the ratio of the peak area in the presence of biological matrix to the peak area in absence of matrix, “pure” solution of the analyte (EMA, 2011). Matrix effect refers to the global impact that the interferences from the evaporated liquid (from LC) have on the process of ionization of the analyte within the ion source region and it has been defined as the Achilles heel of quantitative LC-MS/MS with ESI (Taylor, 2005). When the peak area of the analyte of interest is lower in biological matrix than in the absence of matrix, the term “ion suppression” is used. On the other hand, “ion enhancement” occurs, when an increase of ionization is observed, thus the peak area of analyte in matrix is greater than in “pure” solution (Vogeser and Seger, 2010).
(5) Stability is defined as the chemical stability of an analyte in a given matrix under specific conditions for given time intervals. Stability of the analyte should be ensured in every step of analytical method development, validation and sample treatment.

Coefficient of variation (CV) is used in every single test during the method validation to evaluate the variability of the method. It should be accepted as maximum of 15% for all QCs, except LLOQ, where it should not deviate by more than 20%. The relative recovery and matrix effect need not to be 100%, the range between 80-120% is still acceptable (EMA, 2011; FDA, 2001). Detailed description of each test realized for method validation is summarized in any of the first 4 articles included in this work (Wojnicz et al., 2016a; Wojnicz et al., 2016b; Wojnicz et al., 2013; Wojnicz et al., 2015).

There are 3 main sources of inaccuracy in LC-MS/MS, especially when ESI in positive ionization mode is applied: 1) when the target analyte from complex matrix co-elutes; 2) when isotope-labeled IS is not used; 3) when compounds from the sample matrix share mass transitions with the target analyte. However, in an individual assay, the majority of potential sources of inaccuracy can be controlled by sufficient LC separation (Vogeser and Seger, 2010). Matrix effect is one of the most important validation aspects, since significant matrix effect produces ion suppression in ionization process (Annesley, 2003; Jessome and Volmer, 2006; Muller et al., 2002). Although strategies for successful matrix effect elimination, such as SPE sample preparation, exist (Chambers et al., 2007; Matuszewski et al., 2003; Yaroshenko and Kartsova, 2014), it is necessary to take it into account during the method development and validation. Additionally, carry-over effect and contamination should be considered (Hughes et al., 2007) and, if necessary, dilution integrity, too (EMA, 2011).

Strategies for untargeted method validation

Analytical challenges for untargeted method validation are completely novel and different to the target methods (Naz et al., 2014). These methods require an excellent analytical instrumentation (HPLC and MS). Additional strategies using High Resolution MS (HRMS) can improve sensitivity (e.g. exact mass filtering) and novel databases allow searching compounds related to their molecular formula. New bioinformatics softwares represent a very important challenge in untargeted screening, leading to reports of false positives and false negatives (Hird et al., 2014). Untargeted metabolomic workflow can be divided in 3 main steps:

1) Sample preparation (usually fast and simple)

2) MS data acquisition and processing. The “raw” data must usually be processed, to normalize chromatographic conditions and to carry out accurate quantification of MS features. The features obtained from the processing may be identified later.

3) Statistical analysis. Typically, the statistical analysis includes multivariate techniques, such as supervised and non-supervised methods (Chao De La Barca et al., 2015).

All aspects introduced in this section, such as LC separation, MS as detection, sample preparation and type of analysis (targeted or untargeted) should be carefully taken into account when a bioanalytical method is developed and validated. A correct experimental design will ensure successful method development and help to achieve the best results.

3. LC-MS in clinical pharmacokinetic studies

3.1. Importance of clinical pharmacokinetic studies

Clinical pharmacokinetics is a discipline that describes the absorption, distribution, metabolism and elimination of drugs (ADME of drugs) in patients requiring drug therapy. It explains the time course of a drug concentration in a body fluid (blood, CSF) that results from the administration of certain dosage of the drug (how drugs are handled by the body). Critical factors for choosing the best sort of drug for a patient are age, gender, weight, ethnic background, other concurrent disease states and other drug therapy (Bauer et al., 2011). Thus, the clinical pharmacokinetic studies are necessary and essential for bioavailability and bioequivalence clinical trials (Bobka, 1993), drug discovery (Nishant T, 2011), drug safety evaluation (Eason et al., 1990), drug-drug interactions (Palleria et al., 2013), drug dosage adjustment (Mehrotra et al., 2007) or toxicity evaluation (Sun et al., 2010). Ideally, clinical pharmacokinetic studies can be applied to personalize medicine for chronic diseases (Salzer et al., 2016).

Therapeutic drug monitoring (TDM) by measuring the drug concentration in body fluids (usually in plasma) tends to be applied, because drug measurement directly at the site of drug action is often impossible. One of the main criteria to include a drug in TDM is a good relationship between drug plasma level and efficacy or toxic effect. Other indications include drug-drug interactions, failure or adherence to treatment, narrow therapeutic ranges, drugs with marked pharmacokinetic variability, medications for which target concentrations are difficult to monitor, and drugs known to cause therapeutic and adverse effects (Kang and Lee, 2009). The drug plasma levels need to be interpreted together with patient medical history and could optimize clinical outcomes, such as treatment efficacy and the management of side effects (Ghiculescu, 2008).

Thus, a brief overview of pharmacokinetic parameters will be introduced. To begin with, **Figure 9** shows a typical plasma concentration-time profile after oral drug administration. Drug plasma levels are described with specific pharmacokinetic parameters:

- C_{max} is the maximum plasma concentration of the drug
- t_{max} is the time to reach C_{max}
- C_{min} is the lowest (trough) concentration that a drug reaches before the next dose is administered

- AUC, area under the curve, is a function of the extent of absorption and represents the overall systemic exposure of the drug

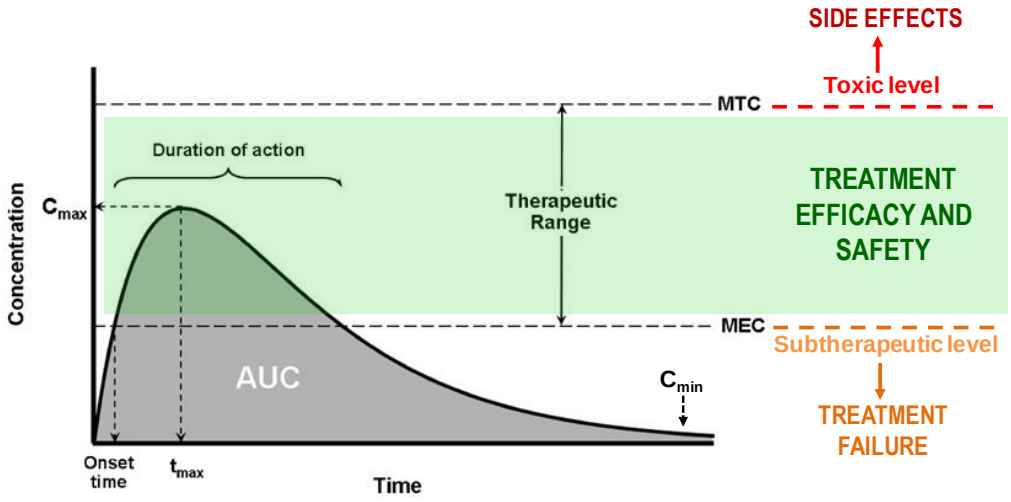

Figure 9. Typical pharmacokinetic parameters describing plasma concentration versus time profile after an oral administration of a drug. Abbreviations: C_{max}: maximum plasma concentration; t_{max}: time to reach C_{max}; C_{min}: minimum plasma concentration AUC: area under the curve; MEC: minimum effective concentration; MTC: maximum tolerated concentration; onset time: the time required for the drug to reach MEC after administration; Definitions: duration of action: the time difference between the onset time and the time for the drug to decline back to MEC; therapeutic range: concentration between MEC and MTC; toxic level: level above MTC; subtherapeutic level: concentration below MEC. Modified from Mehrotra et al., 2007.

Therapeutic range of a drug is the range of drug concentration when treatment efficacy is reached and no significant side effects are observed. However, when drug concentration overcomes the maximum tolerated concentration (MTC), toxic levels are reached and side effects may appear. Conversely, when the drug does not achieve minimum effective concentration (MEC) needed to produce pharmacological effect, subtherapeutic levels are observed and the risk of treatment failure is increased (Mehrotra et al., 2007). Thus, in many cases TDM is required for dose adjustment in order to minimize adverse effects and achieve treatment efficacy.

In this work, I am going to demonstrate the importance of MS in 2 selected clinical pharmacokinetic applications of the following drugs:

- ABZ and its main metabolite albendazole sulfoxide (ABZOX)
- OME

Two LC-MS/MS methods for ABZ/ABZOX and OME plasma determination have been successfully developed, validated and applied for clinical pharmacokinetic bioequivalence studies in Clinical Trials Unit, Department of Clinical Pharmacology,

Hospital Universitario de la Princesa, Madrid. Original publications are provided in the "Original Articles" section.

3.2. Albendazole and albendazole sulfoxide

ABZ, 5-(propylthio)-2-carbomethoxyaminobenzimidazole, is a bezimidazole derivative, an oral broad-spectrum anthelmintic agent. The principal mode of action for albendazole is by its inhibitory effect on tubulin polymerization, resulting in a loss of cytoplasmatic microtubules. ABZ is used for treatment of parasitic infections, such as neurocysticercosis. It is the most common helminthic disease of central nervous system (CNS) caused by larval forms of the pork tapeworm, *Taenia solium*, that affect developing population of Latin America, Asia and Africa (Del Brutto, 2005; Flisser et al., 2003; Takayanagui, 2004). ABZ is also used for human cystic hydatid disease of the liver, lung, and peritoneum, caused by the larval form of the dog tapeworm, *Echinococcus granulosus* (Cobo et al., 1998; Horton, 1997; Lipani et al., 1997; Mohamed et al., 1998; Yasawy et al., 1993), often in combination with praziquantel (Cobo et al., 1998; Mohamed et al., 1998; Yasawy et al., 1993). After oral administration, during the absorption process, ABZ is rapidly metabolized to its main and active metabolite ABZOX and further transformed to an inactive metabolite albendazole sulfone (Dayan, 2003; Marques et al., 2002; Marriner et al., 1986; Rawden et al., 2000). **Figure 10** shows the chemical structure of ABZ and its main metabolite ABZOX. ABZOX is a mixture of 2 enantiomers: in animals *R*(+) is catalyzed by microsomal flavin monooxidase and *S*(-) is metabolized by cytochrome P450 enzymes (CYP), particularly by CYP3A (Delatour et al., 1991; Moroni et al., 1995). *R*(+) enantiomer is CYP2D6 substrate and it seems to be predominant in humans (Drugbank). Plasma concentration of ABZ after oral administration is often very low. Thus, pharmacokinetic properties of ABZ have been studied by determination of ABZOX plasma levels (Jung et al., 1992; Mirfazaelian et al., 2002; Takayanagui et al., 1997), present in order of ng/mL. ABZ is poorly absorbed (about 30%) from the gastrointestinal tract due to its low aqueous solubility, which often leads to treatment failure. Thus, TDM is necessary for optimizing treatment in resistant patients (Castro et al., 2009) and for improvement of drug therapy (Skuhala et al., 2014).

ABZ **ABZOX**

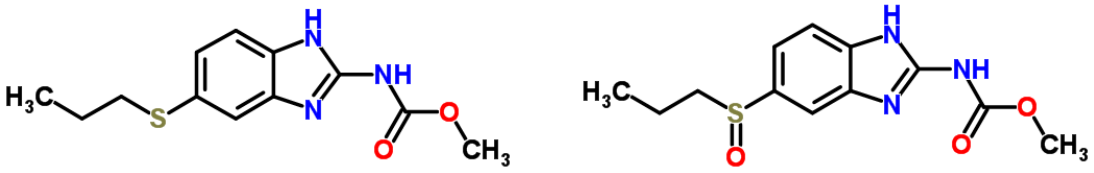

Figure 10. Chemical structure of ABZ and ABZOX. Abbreviations: ABZ: albendazole; ABZOX: albendazole sulfoxide. Taken from ChemSpider.

Several analytical methods have been reported in the literature for human plasma quantification of ABZ and its main metabolite, ABZOX. HPLC coupled to FLD detector (HPLC-FLD), HPLC-UV or even CE methods were often used for ABZ and ABZOX plasma monitoring (Garcia et al., 1999; Hurtado et al., 1989; Kitzman et al., 2002; Lanchote et al., 1998; Mirfazaelian et al., 2002; Prochazkova et al., 2000; Sarin et al., 2004). However, in the last 15 years, LC-MS/MS based methods have been often the first choice for the ABZ and/or ABZOX plasma determination, due to their better sensitivity and selectivity (Bonato et al., 2007; Bonato et al., 2003; Chen et al., 2004; Gonzalez-Hernandez et al., 2012; Saraner et al., 2016; Wojnicz et al., 2013). For sample preparation, different extraction methods, such as PPT, LLE or SPE have been applied (Bonato et al., 2007; Chen et al., 2004; Gonzalez-Hernandez et al., 2012; Hoaksey et al., 1991; Hurtado et al., 1989; Kitzman et al., 2002; Prochazkova et al., 2000; Saraner et al., 2016; Sarin et al., 2004).

In the present work, a simple, selective and sensitive LC-MS/MS method for simultaneous determination of ABZ and ABZOX in human plasma was successfully developed and validated: “A simple assay for the simultaneous determination of human plasma albendazole and albendazole sulfoxide levels by high performance liquid chromatography in tandem mass spectrometry with solid-phase extraction“(Wojnicz et al., 2013). The present assay has been applied for pharmacokinetic studies of ABZ and ABZOX, in 12 healthy volunteers with oral ABZ dosis of 400 mg/d, which results will permit to use it in clinical trials and routine clinical practice.

3.3. Omeprazole

One of the 10 most prescribed medications, prodrug OME (Weinstock and Johnson, 2016), with chemical nomenclature 6-metoxy-2-[(4-methoxy-3,5-dimethyl-2-pyridinyl)methylsulfinyl]-*H*-benzimidazole, is a selective and potent inhibitor of gastric acid secretion by parietal cells. OME acts through H^+/K^+-ATPase (hydrogen/potassium ATPase, gastric enzyme) inhibition (Wallmark et al., 1983). **Figure 11** shows the

chemical structure of OME. In the treatment of infections produced by *Helicobacter pylori*, stomach ulcers, gastroesophageal reflux disease and Zollinger-Ellison syndrome, OME was the first line treatment of all proton pump inhibitors (Clissold and Campoli-Richards, 1986; Robinson and Horn, 2003; Sachs et al., 2006). The bioavailability of OME has been studied for single oral dose of 40 mg and is described as 58% (Clissold and Campoli-Richards, 1986; Robinson and Horn, 2003; Sachs et al., 2006). Nowadays, the bioavailability with low oral dose of 100-200 µg is intensively investigated, to minimize the risk of adverse effects in human subjects (Oh et al., 2012; Sugiyama and Yamashita).

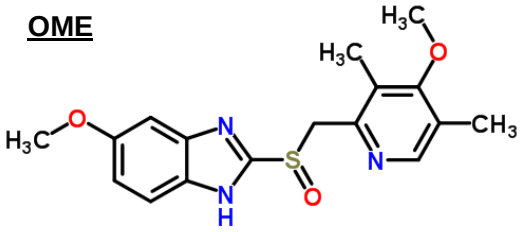

Figure 11. Chemical structure of OME. Abbreviation: OME: omeprazole. Taken from ChemSpider.

OME is metabolized by CYP to 2 main metabolites. 5-hydroxyomeprazole (H-OME) is mainly formed by CYP2C19 and omeprazole sulfone (OME-S) by CYP3A4 (Andersson et al., 1994; Chiba et al., 1993). The genotype of *CYP2C19* significantly determines the pharmacokinetics of OME (Cederberg et al., 1989). Because of cytochrome P450 metabolism, OME produces significant drug-drug interactions, in some cases inhibits the metabolism of certain drugs (Blume et al., 2006; Wedemeyer and Blume, 2014) and is one of the drugs causing interactions in dental treatment (Weinstock and Johnson, 2016). As OME pharmacokinetic is strongly genotype and drug-drug interactions dependent, there is a strong need of TDM for OME. Thus, the pharmacokinetic studies are necessary for dose adjustment and will help to understand drug-drug interactions, particularly in polymedicated patients.

To date numerous methods have appeared in the literature for OME and/or its main metabolites determination in human plasma. HPLC-UV, HPLC coupled to coulometric detection, HPLC-MS or even CE with UV detection (CE-UV) have been used for OME quantification (Ahmed and Atia, 2015; Bharathi et al., 2009; Garcia-Encina et al., 1999; Gonzalez et al., 2002; Nevado et al., 2014; Noubarani et al., 2010; Perez-Ruiz et al., 2006; Shimizu et al., 2006; Shiohira et al., 2011; Sluggett et al., 2001; Yuen et al., 2001; Zarghi et al., 2006). However, LC-MS/MS offers more sensitivity, selectivity and significantly shorter time of analysis and thus many authors

choose this technique for analytical laboratory (Dodgen et al., 2011; Frerichs et al., 2005; Macek et al., 2007; Oh et al., 2012; Song and Naidong, 2006; Vittal et al., 2009; Woolf and Matuszewski, 1998). More recently, a combination of fully automated validated LC-MS/MS method and Real Time-PCR analysis was applied for pharmacokinetic and pharmacogenetic studies of OME in healthy volunteers (Koukoula et al., 2016). For the sample preparation, different extraction methods, such as PPT, LLE and SPE have been applied (Ahmed and Atia, 2015; Bharathi et al., 2009; Frerichs et al., 2005; Garcia-Encina et al., 1999; Gonzalez et al., 2002; Macek et al., 2007; Nevado et al., 2014; Noubarani et al., 2010; Oh et al., 2012; Shimizu et al., 2006; Shiohira et al., 2011; Vittal et al., 2009; Woolf and Matuszewski, 1998; Yuen et al., 2001). Lately, automated LLE (Koukoula et al., 2016; Song and Naidong, 2006) and SPE (Dodgen et al., 2011; Perez-Ruiz et al., 2006) are also being used because of their time and bias reduction compared to manual sample preparation.

In the present work, we report an improvement and validation of LC-MS/MS for OME quantification in human plasma: “Improvement and Validation of a High-Performance Liquid Chromatography in Tandem Mass Spectrometry Method for Monitoring of Omeprazole in Plasma” (Wojnicz et al., 2015). The current method is aimed to provide a time saving approach for pharmacokinetic and drug-drug interactions studies in healthy volunteers and patients. The validated method was successfully applied for OME pharmacokinetics in 240 real samples from 6 healthy volunteers, after oral administration of OME (Losec, 40 mg/d) and under fasting conditions. The samples for pharmacokinetic curves were collected before OME administration and up to 12 h after administration.

4. LC-MS in neurotransmitter research

4.1. Neurotransmitters and their functions

Neurotransmitters (NTs) are essential bioactive molecules in CNS and peripheral body fluids of mammals. NTs play an important role in physiological pathways of nervous system, but are also involved in hormone metabolism (Webster, 2002). It has been thought for many decades that one neuronal cell releases one neurotransmitter. Nowadays, co-transmission is widely accepted as a habitual process in central and peripheral nervous system (Gutierrez, 2008; Hnasko and Edwards, 2012). Many NTs are released at the same time, acting as inhibitors and/or activators of certain neurophysiological processes (Gutierrez, 2008). Until now, 5 types of neurotransmitters are known (Webster, 2002):

- choline ester: acetylcholine (ACh)
- biogenic monoamines: catecolamines (adrenaline [AD], noradrenaline [NA] and dopamine [DA]), serotonin (5-HT) and histamine (HIST)
- amino acids: γ-aminobutyric acid (GABA), glutamic acid (Glu) and glycine
- nucleotides: adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), cyclic adenosine 5′-monophosphate (cAMP)
- neuropeptides: enkephalines (methionine-enkephalin [MENK] and leucine-enkephalin [LENK]), endorphins, cholecystokinin and substance P

The NTs metabolites also play an important role in neurotransmission, and **Figure 12** shows 4 different groups of NTs that have been addressed in the methods described in articles 3, 4 and DiB: monoamines in red (AD, NA, DA, 5-HT, HIST); amino acids in black (GABA, Glu); neuropeptides in green (MENK, LENK); nucleotides in dark blue (ADP, AMP, cAMP) and some of their metabolites, such as metanephrine (MN), metabolite of AD; 3-Methoxy-4 hydroxyphenylglycol (MHPG), metabolite of AD and NA; and 5-hydroxyindoleacetic acid (5-HIAA), metabolite of 5-HT, in violet.

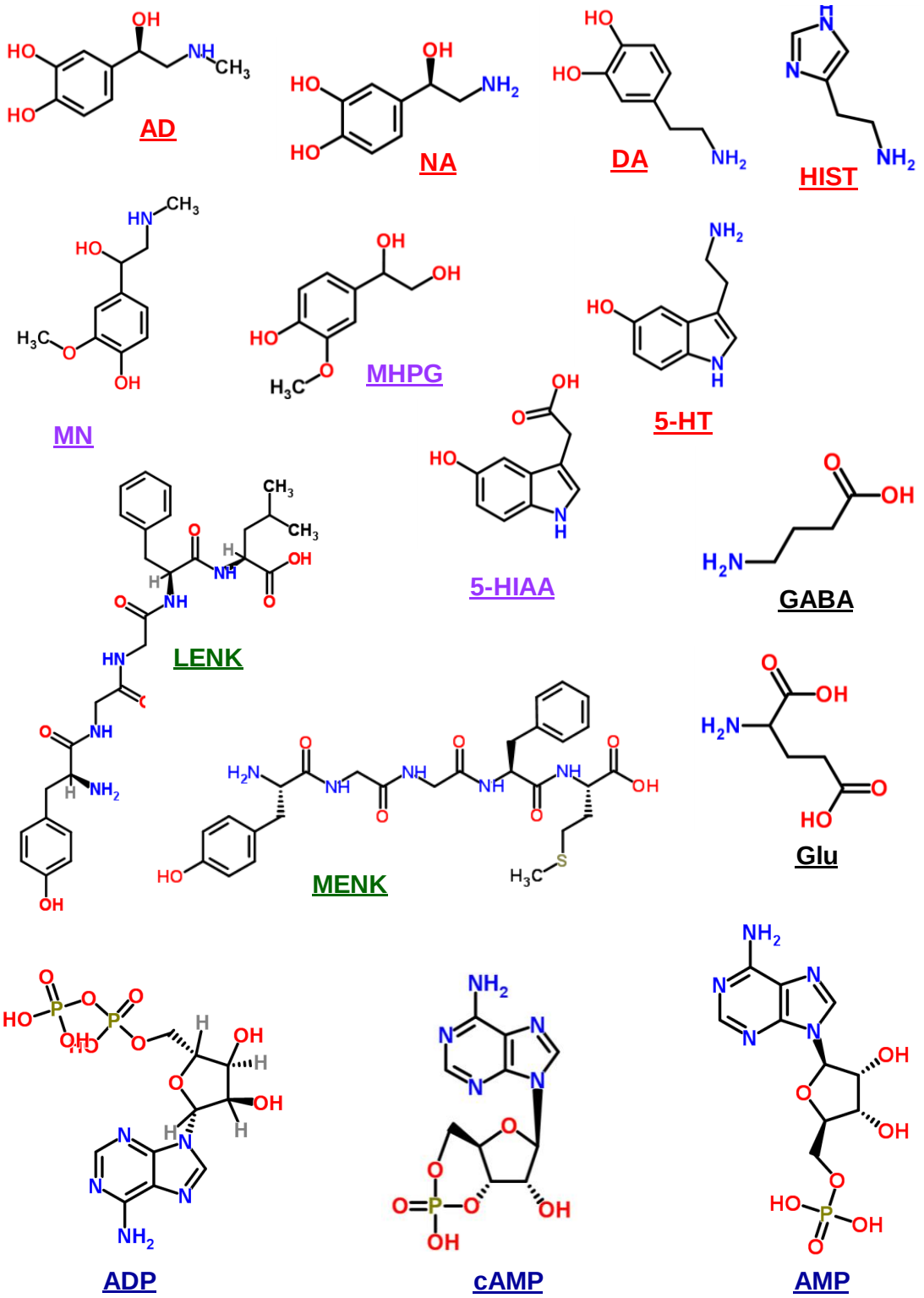

Figure 12. Chemical structure of 4 groups of neurotransmitters. Monoamines (in red), amido acids (in black), nucleotides (in dark blue), neutopeptides (in green) and some of their metabolites (in violet). Abbreviations: AD: adrenaline; NA: noradrenaline; DA: dopamine; 5-HT: serotonin; MN: metanephrine, metabolite of AD; MHPG: 3-Methoxy-4 hydroxyphenylglycol, metabolite of NA and AD; 5-HIAA: 5-hydroxyindoleacetic acid, metabolite of 5-HT; HIST: histamine; GABA: γ-aminobutyric acid; Glu: glutamic acid; MENK: methionine-enkephalin; LENK: leucine-enkephalin; ADP: adenosine 5′-diphosphate; AMP: adenosine 5′-monophosphate; cAMP: cyclic adenosine 5′-monophosphate. Taken from ChemSpider.

Neurotransmission is involved in many processes of CNS, such as memory, learning, anxiety, pain and behavior (Webster, 2002). Thus, alteration of NTs brain levels can lead to various pathological processes. In human and animal models, NTs alteration has been associated with neurodegenerative diseases, such as Alzheimer's or Parkinson's disease (Advokat and Pellegrin, 1992; Stahl, 2008; Tsunoda et al., 2010) and other neuropathophysiological disorders, including schizophrenia, epilepsy and Down syndrome (Carlsson et al., 1997; Meldrum, 1984; Seidl et al., 1999; Stahl, 2008; Treiman, 2001; Tsunoda et al., 2010). However, NTs role as biomarkers still remains to be clarified (He et al., 2016; Lang et al., 2004). Neuropsychiatric disorders, such as depression have been associated with decrease of monoamine NTs, in particular 5-HT and NA (Schildkraut, 1965). Thus, diagnostic biomarkers of neuropsychiatric diseases (Su et al., 2009; Zheng et al., 2012) and antidepressant drug efficacy (Yoshitake et al., 2003) have been investigated to improve the treatment. Neurochemical relationship between neurodegenerative diseases, such as dementia, Alzheimer's and Parkinson's diseases and major depressive disorder has recently been investigated and strong associations have been found (Kim et al., 2016; Reus et al., 2016). Changes in neuroplasticity, morphology and neurotransmission (Reus et al., 2016), such as Glu and GABA in the brain (Kim et al., 2016), are common for major depressive disorder and neurodegenerative diseases. Diagnosis and treatment strategies of neurodegenerative and neuropsychiatric diseases are complicated. Combination of clinical biomarkers (genotype, blood and CSF analyte), brain imaging, cognitive assessment, and medical history data, may thus help to establish earlier diagnosis and improve the treatment (Ramanan and Saykin, 2013). MS is currently the most sensitive technique for searching of NTs biomarkers in tissue. IMS has been recently applied for brain imaging. A novel desorption-electrospray ionization IMS (DESI-IMS) method has been developed and is able to monitor NTs and neuroactive substances in native rat and mouse brain (Shariatgorji et al., 2016).

Neurotransmission is also involved in peripheral nervous system. NTs are present in peripheral body fluids. When NTs from adrenal gland are altered, neuroendocrine diseases, like pheochromocytoma, paraganglioma and neuroblastoma can appear. Abnormally high concentration of endogenous catecholamine NTs in plasma and urine are used as biomarkers for these diseases (Goldstein et al., 2003; Kushnir et al., 2002). For example, clinical research on foetal adrenal gland to study Parkinson's disease have been performed (Garcia et al., 1994). Nevertheless, in order to better understand normal and pathophysiological pathways (disease, stress conditions), basic neuroscience studies are needed, including exocytosis and

endocytosis processes on bovine chromaffin cells (BCCs) cultures (Carrera et al., 2007; Muller and Unsicker, 1981). One of the stress conditions is the exposure to metal toxicity which has been associated with neurotoxicity and urine monoamine metabolite changes in Alzheimer's disease (Double et al., 1998; Manini et al., 2000). Thus, MS gives an excellent opportunity for body fluid monitoring in search for NTs biomarkers and improve the diagnosis of NTs-associated diseases.

4.2. Rat brain neurotransmitters and murine depression model

Depression is a neuropsychiatric disease and the most accepted theory on its pathogenesis is known as monoamine theory proposed by Schildkraut in the 60s (Schildkraut, 1965). This hypothesis describes a deficit of NTs, NA and 5-HT in the brain. The mechanism of action of the most common treatments (e.g., tricyclic antidepressants and 5-HT reuptake inhibitors) is through the inhibition of reuptake and metabolism of these NTs in neuronal synapses. More recently, a deficit in GABA neurotransmitter has been found to be involved in major depressive disorder and anxiety disorders (Luscher et al., 2010; Treadway and Pizzagalli, 2014; Treadway and Zald, 2011). **Figure 13** displays schematic alterations in monoamine levels and receptor availability as well as alterations in Glu and GABA in human brain. For this reason, monitoring of NTs and their metabolites in the CNS of animal models is fully justified as an important tool for understanding the pathophysiology of depression and to evaluate the pharmacological effect of possible treatments.

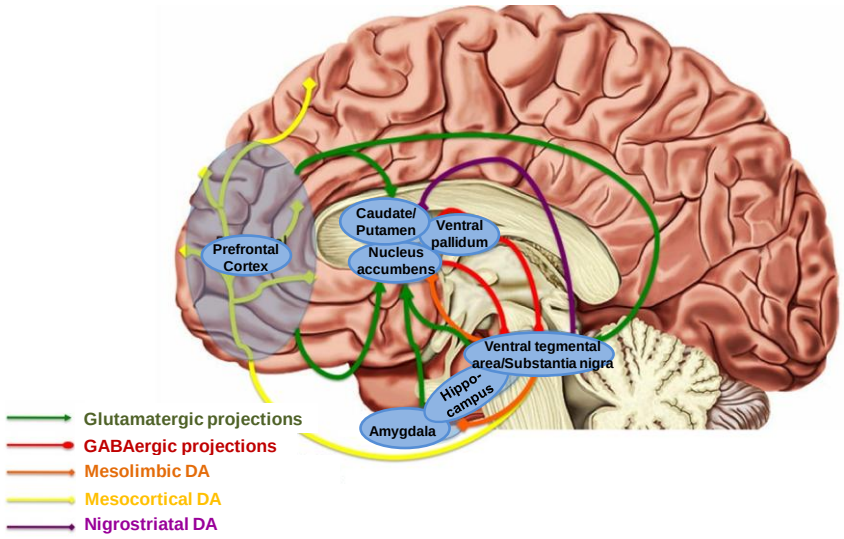

Figure 13. Regions, NTs and circuits implicated in the pathology of major depressive disorder by human neuroimaging studies. Past studies have identified alterations in monoamine levels and receptor availability as well as alterations in Glu and GABA. These neurotransmitter systems participate in larger circuits involved in the experience and regulation of emotion, responses to stress, and processing of rewards. Note: placement of structure labels is approximate. Abbreviations: GABA: γ-aminobutyric acid; Glu: glutamic acid; NTs: neurotransmitters. Reproduced and modified from Treadway and Pizzagalli, 2014 and Treadway and Zald, 2011.

There are many reports relating depression with NTs changes in CNS in rat brain models (Ding et al., 2016; Green and Grahame-Smith, 1978; Ruda-Kucerova et al., 2015; Smolders et al., 2008). However, only few reports deal with the depression in mouse brain model (Freitas et al., 2016; Martin-de-Saavedra et al., 2013). There are 2 different models of depression in mice:

- Corticosterone-induced depression model: Zhao and colleagues (Zhao et al., 2008) found that repeated corticosterone injection provides a useful and reliable mouse model of a depressive illness
- Nuclear factor (erythroid 2-derived)-like 2 (Nrf2) depression model: Study from Martin-de-Saavedra and colleagues (Martin-de-Saavedra et al., 2013) revealed that Nrf2 model can be used as a depression model. Chronic inflammation due to a deletion of Nrf2 gene can lead to a depressive-like phenotype, while induction of Nrf2 could become a new and interesting target to develop novel antidepressive drugs.

Accordingly, we proposed an interesting application of our simple assay of 8 NTs and their metabolites, in order to study NTs changes in mouse Nrf2 model of depression (Wojnicz et al., 2016b).

4.3. Bovine chromaffin cells from adrenal gland

Chromaffin cells are the secretory cells from the adrenal medulla that secrete catecholamines (AD, NA and DA). Catecholamines play an important role in stress response (Kopin, 1976), known as the “fight or flight” response. For years, they served as a valuable model for basic mechanisms involved in calcium (Ca^{2+}) signaling and exocytosis (Garcia et al., 2006). AD and NA released in the exocytotic process were classically used for neurosecretion studies *in vitro*. BCCs have been applied for chromaffin vesicles isolation and characterization of their compounds (Winkler and Westhead, 1980), because of their easy cultivation and unlimited availability (Livett, 1984). The versatile and multifunctional chromaffin vesicles include not only catecholamines and opiates, such as LENK and MENK (Crivellato et al., 2008; Winkler, 1993), but also ATP, which is co-stored with catecholamines in 1 to 4 ratio (Castillo et al., 1992; Sillero et al., 1994; Todorov et al., 1996). Other NTs observed in chromaffin vesicles have been studied more recently and are represented by nucleotides: ADP, AMP and cAMP; excitatory and inhibitory amino acids: Glu and GABA; and other monoamines, such as 5-HT and its main metabolite, 5-HIAA; HIST and MN, the metabolite of AD. The total intracellular content of catecholamines has been defined previously in bovine adrenal medulla and consist of 60-70% adrenergic and 30-40% noradrenergic cells (Moro et al., 1990). The amount of these NTs in chromaffin granules is very high and they could therefore be detected with previous, less sensitive, assays. For decades it was thought that GABA and Glu are present only in CNS. However, amino acids are also present in BCCs, though their concentration in BCCs is lower compared with CNS. GABA and Glu were investigated for the first time in 2003 in a study by Romero and colleagues, where the release of Glu from BCCs stimulated cells was reported (Romero et al., 2003). Then, the existence, storage and release of GABA in chromaffin granules have also been described in 2010 by the Harada group (Harada et al., 2010). **Figure 14** shows the storage and signaling of GABA in large dense core vesicles (LDCVs) or chromaffin granules in chromaffn cells of rat adrenal chromaffin cells (Harada et al., 2016).

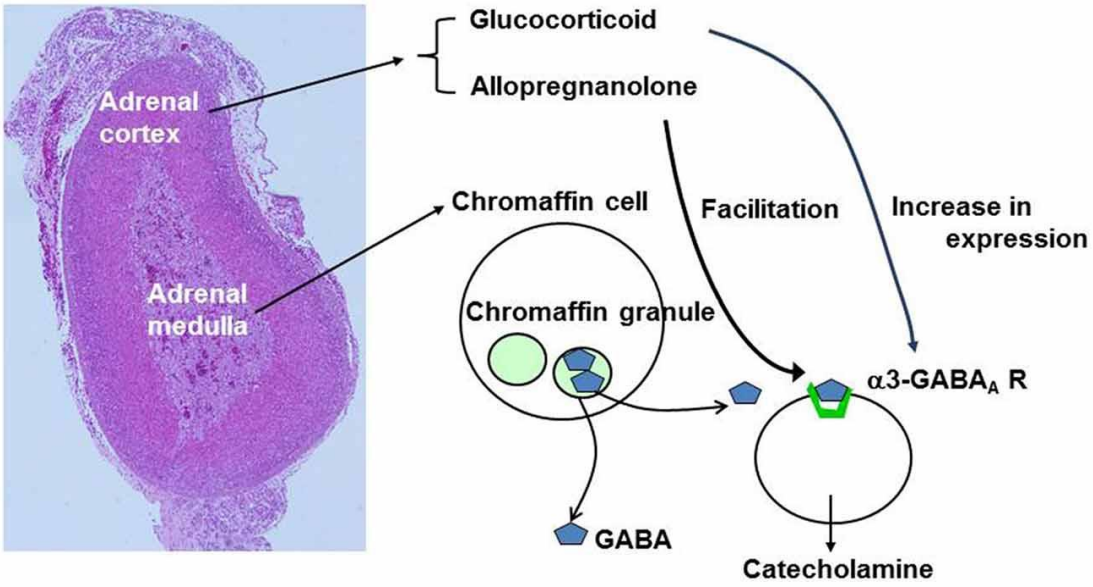

Figure 14. Summary of effects of adrenal cortical hormones on GABA signaling. The left image represents hematoxylin-eosin staining of mouse adrenal gland. GABA is stored in LDCVs or chromaffin granules in chromaffin cells. Glucocorticoids produce an increase in expression of α3-GABA$_A$ R, whereas allopregnanolone produces facilitation of GABA$_A$ receptor Cl^- channel activity. Abbreviations: α3-GABA$_A$ R: α3-containing GABA$_A$ receptors; GABA: γ-aminobutyric acid; LDCVs: large dense core vesicles. Taken from Harada et al., 2016.

In order to understand the complexity of the neurosecretion process many different assays have been developed. In the present part of the work, a brief overview on LC-MS based methods for NTs research is provided. NTs were determined in biological fluids, tissue and cell cultures. Classically, catecholamines have been measured with ED because of their redox potential. However, ED detection without a separation technique provides global information about all catecholamines present in the sample (AD, NA and DA) without differentiation. For this reason, it is necessary to use previous analyte separation through HPLC before ED detection (HPLC-ED). Commercial kits for CT plasma determination are available. However, they are often expensive and only routine analytical laboratory should use them as a daily solution. In the literature, numerous methods using HPLC-ED for biogenic monoamines and their metabolites and/or neuropeptide quantification have been described (Chritton et al., 1997; Del Pino et al., 2011; Kotake et al., 1985; Muller and Unsicker, 1981; Murai et al., 1988; Nguyen et al., 2010; Parrot et al., 2011; Wang et al., 2008; Xu et al., 2002). However, these methods are unable to detect extreme polar compounds, such as GABA and Glu. AMP, ADP and ATP are also extremely polar compounds due to the presence of multiple phosphate groups which may interfere with ED. Because of their physical and chemical properties amino acids and nucleotides tend to coelute and produce very similar RT. Some authors have used HPLC-UV or HPLC-FLD methods for monoamines (and some of their metabolites), nucleotides and amino acid

determination (Davis et al., 1978; Ushimaru and Fukushima, 2003; Wu et al., 2013; Yoshitake et al., 2003). The disadvantage of ED, UV and FLD is lower sensitivity and longer separation time. Other methods, such as radioimmunoassay (RIA), have been used for monoamines and neuropeptide determination (Podvin et al., 2015). However, the RIA is an expensive assay and does not permit simultaneous determination of more than one analyte in a single experiment. LC-MS/MS offers greater sensitivity and sensibility than other methods (Bicker et al., 2013). MS detection is able to identify compounds with very similar RT, due to its *m/z*, specific for each single compound. This technique permits determination of molecules with different physical-chemical properties in only one analytical run, which makes the analysis shorter and improves effectiveness. Numerous methods using LC-MS/MS or UPLC-MS/MS have been developed for quantification of monoamines and/or its metabolites, and/or amino acids, and/or neuropeptide and/or nucleotide NTs in tissue and body fluids (Bergh et al., 2016; Buck et al., 2009; Cai et al., 2009; Carrera et al., 2007; Gonzalez et al., 2011; Gu et al., 2008; He et al., 2013; Lang et al., 2004; Qian et al., 2004; Su et al., 2009; Tareke et al., 2007; Xu et al., 2011; Zhu et al., 2011). Recently, a novel DESI-IMS method has been used for monoamines, their metabolites and amino acid NTs in native rat brain tissue (Shariatgorji et al., 2016). Unfortunately, none of those methods is able to simultaneously quantify 4 groups of NTs: monoamine, amino acids, neuropeptides and nucleotides.

PPT, LLE and SPE have been used for analyte extraction from tissue, body fluids or cells (Bergh et al., 2016; Cai et al., 2009; Davis et al., 1978; Del Pino et al., 2011; Gonzalez et al., 2011; Gu et al., 2008; He et al., 2013; Lang et al., 2004; Parrot et al., 2011; Qian et al., 2004; Wu et al., 2013; Yoshitake et al., 2003; Zhu et al., 2011). Lately, more advanced sample preparation, such as micro-LLE or semi-automated SPE have been applied (He et al., 2016; Tareke et al., 2007; Xu et al., 2011). Direct injection to the LC-MS/MS system, avoiding sample preparation (Zhang et al., 2012), have also been described.

NTs monitoring is needed for better understanding of physiological and pathological processes of central and peripheral nervous system. Many times, the determination of analytes representing different physical and chemical properties requires longer time of sample preparation and more than one analytical method (Davis et al., 1978; He et al., 2013; Wu et al., 2013; Yoshitake et al., 2003). However, some molecules, such as AD, NA and DA are easier to oxidate and are unstable (Carrera et al., 2007; Chritton et al., 1997; Muller and Unsicker, 1981; Podvin et al., 2015; Qian et al., 2004; Wojnicz et al., 2016a). Therefore, it is highly recommended to perform a

simultaneous, short and efficient sample preparation method in only one analytical run. Hence, we propose 2 simple LC-MS/MS assays for:

1) Simultaneous monitoring of 8 NTs and some of their metabolite (AD, NA, DA, 5-HT, GABA, Glu, 5-HIAA, MHPG) in rat brain and its application to murine Nrf2 model of depression. Original publication is entitled as below: “Simultaneous determination of 8 neurotransmitters and their metabolite levels in rat brain using liquid chromatography in tandem with mass spectrometry: Application to the murine Nrf2 model of depression“ (Wojnicz et al., 2016b) and DiB related to this article is provided (Wojnicz et al., 2016c).

2) Simultaneous determination of 14 NTs (AD, NA, DA, 5-HT, HIST, GABA, Glu, 5-HIAA, MN, LENK, MENK, ADP, AMP, cAMP) in BCCs and its application to neurosecretion studies under stress conditions. Original article is entitled as follows: “Simultaneous monitoring of monoamines, amino acids, nucleotides and neuropeptides by liquid chromatography-tandem mass spectrometry and its application to neurosecretion in bovine chromaffin cells“ (Wojnicz et al., 2016a).

Both original publications (3 and 4) and the DiB to 3rd article are provided in the “Original Articles” section.

These methods show beneficious applications with the purpose of better understanding physiological and pathological neurosecretion processes and improves the actual knowledge in the field.

5. Imaging mass spectrometry in human habitats studies

5.1. IMS for visualizations of molecules

IMS technique provides molecular mass and spatial information for visualizing molecules in original sample and often complex surfaces. MALDI-TOF MS was first used in 1997 to visualize the spatial distribution of molecules (Caprioli et al., 1997). Other IMS methods, such as desorption ESI MS (DESI-MS), nanospray desorption electrospray ionization-MS (nanoDESI-MS) and secondary ion MS (SIMS) exist, however all of them provided two-dimensional (2D) spatial information about analytes of interest. Thus, in the last part of the thesis, the 5th publication describes an example of a three-dimensional (3D) surface-IMS (3D-surface-IMS) untargeted metabolomics method and its application for modern human habitats (Petras et al., 2016).

5.2. Human habitat studies

Nowadays, many people around the world are living in cities and urbanizations. There is a continuous interaction between the world and us: cosmetics we use, the cars we drive, the homes we live in, etc. All these interactions produce continuous chemicals exchange. However, we do not have a complete knowledge about the chemistry of our environment and how our own chemicals influence the modern human habitat and *vice versa*. Human skin is a “barrier” between the body and the outside world. For this reason, human skin, with a surface area of 1.5-2 m^2, provides the interaction with personal care products, environment and transportation of the modern world. Modern world, especially big cities with significant pollution, includes a large amount of toxic chemicals to the humans and animals. In 2005, Wild (Wild, 2005) introduced the “exposome” concept to explain the sum of possible human environmental exposures. Lately, environmental disease risk factors, including harmful molecules, have been investigated with more intensity (Board-on-Life-Sciences, 2016; Nieuwenhuijsen, 2016; Putignani and Dallapiccola, 2016; Siroux et al., 2016). Personal care products used daily in our modern clean-life style include additives such as triclosan or cocamidopropyl betaine (CAPB) and its impurities, which were shown to have a negative effect on reproduction (Geer et al., 2016; Stoker et al., 2010) and can also cause skin allergies (Suuronen et al., 2012; Zirwas and Moennich, 2009). On top of that, it has been demonstrated that phthalates, used for plastic production, caused developmental and reproductive toxicity (Boisvert et al., 2016; Dobrzynska, 2016; Giudice, 2016; Komada et al., 2016; Whyatt et al., 2011). Polybrominated phenyl ethers, used in every sector of the modern life to prevent fire, have shown disruption of

neurobehavior in rats (Kuriyama et al., 2005). Artificial sweeteners including aspartame, chosen by many people as an alternative to sugar in healthy diet, can produce behavior alterations (Onaolapo et al., 2016) and should be used with precaution because of their possible toxicity (Sharma et al., 2016). Organophosphate flame retardants (OPFRs) (Zhou et al., 2016), polycyclic aromatic hydrocarbons (PAHs) (Han et al., 2015; Singh et al., 2016) and tobacco smoke (Fu et al., 2016), present in indoor air microenvironments, have a negative influence to the public health. In addition, the association between PAHs and kidney dysfunction (Singh et al., 2016) or cancer risk (Han et al., 2016) has recently been investigated. A low quality of the indoor air often produces allergies (Singh and Hays, 2016) and eye irritation (Wolkoff, 2016). All of these interactions between human and their environment and *vice versa* bring a strong evidence for the Locards's exchange principle, "every contact leaves a trace", coined in 1930s (Locard, 1930). Much emphasis has been placed on targeted analysis or monitoring of harmful or potentially harmful molecules in the environment. However, unknown substances in our environment remain undiscovered. Untargeted metabolomics is an excellent tool for studying the chemistries associated with the human habitat. It enables the investigation of human chemicals and chemicals provided by environment, and their exchange between humans and the surrounding environment.

MS is a sensitive approach that has been used for human and environmental studies. However, the conventional HPLC-MS or gas chromatography-MS is limited to the spatial distribution of the sample, needed for this study. Thus, IMS, enabling the spatial information of the sample, has become fairly common in the last decade.

Table 2 shows targeted and untargeted imaging mass spectrometric methods in the literature. 2D MS offers very interesting applications, including spatiotemporal dynamics of microbial metabolites production (Watrous et al., 2012), spatial drug distribution studies (Bokhart and Muddiman, 2016), spatial differences in chemistries of aquatic environment research (Rivas et al., 2016). Even 2D MS has been applied to map the presence of metals and other elements in thin tissue sections of post-mortem cases (Lauer et al., 2016). However, described IMS, such as MALDI TOF-MS, DESI-MS, nanoDESI-MS and laser ablation inductively coupled plasma MS (LA-IPC-MS), has been limited to small sample size (from 5 mm to few cm) and spatial resolution between 10-100 µm. Secondary ion MS (SIMS) offers better resolution, up to 30 nm (Jungnickel et al., 2016). Although this is an important advantage comparing to conventional MS, it is still not sufficient for analysis of the human body, human environment and transportation (Caprioli, 2016; Rivas et al., 2016; Watrous and

Dorrestein, 2011; Wu et al., 2012b). For the analysis of this kind of profile cm or m are needed, to give 3D sample information.

Analytes	Sample localization	Application	Method	Author
Microbial metabolites	Live microbial colonies	Spaciotemporal dynamics of metabolites production	**2D nanoDESI-MS**	(Watrous et al., 2012)
Organosulfates	Urban areas	Contamination information of urban areas	**2D nanoDESI-MS**	(Tao et al., 2014)
Chemical compounds or biomolecules	Food	Food science and related fields	**2D MALDI-MS**	(Yoshimura et al., 2016)
Products of solid polymers	Water (aerobic and denitrifying conditions)	Aquatic environment research	**2D MALDI TOF-MS**	(Rivas et al., 2016)
Several drugs	Biospecimes; Tissue sections	Spatial drug distribution and metabolomics	**2D IR MALDESI-MS**	(Bokhart and Muddiman, 2016; Nazari et al., 2016)
Metals and other elements	Tissue sections of post-mortem cases	Forensic pathology and toxicology	**2D LA-IPC-MS**	(Lauer et al., 2016)
Polyethylene microplastics	Sand	Eco-toxicology and human health risk assessment	**2D TOF-SIMS**	(Jungnickel et al., 2016)
Microbial metabolites exchange	Live microbial colonies	Behavior and interspecies interactions of neighboring microbes	**3D MALDI TOF-MS**	(Watrous et al., 2012)
Chemical “make-up” of skin molecules and bacteria	Human skin surface	Relationship of human skin with hygiene, microbiota, and environment	**3D-surface-IMS**	(Bouslimani et al., 2015)
Small molecules and metabolites	**Human skin and human environment**	**Chemistry of modern human habitat**	**3D-surface-IMS**	***(Petras et al., 2016)**

Table 2. Targeted and untargeted imaging mass spectrometric methods in the literature. Abbreviations: 2D: two-dimensional; 3D: three-dimensional; nanoDESI-MS: nanospray desorption electrospray ionization-mass spectrometry; MALDI-MS: Matrix-assisted laser desorption/ionization mass spectrometry; MALDI TOF-MS: MALDI time-of-flight mass spectrometry; IR MALDESI-MS: Infrared matrix-assisted laser desorption electrospray ionization mass spectrometry; LA-ICP-MS: Laser ablation inductively coupled plasma mass spectrometry; TOF-SIMS: Time-of-flight-secondary ion mass spectrometry; 3D-surface-IMS: three-dimensional-surface-imaging mass spectrometry. *The method we developed is highlighted in green.

Bouslimani and co-workers (Bouslimani et al., 2015) have designed a 3D cartography method which translates MS features onto a 3D model of the sample under investigation. In this way, many samples are taken spatially into account. This strategy enables analysis of large spatial spaces, such as human body. **Figure 15** shows spatial distribution of molecular profiles, using 3D-surface-IMS technique. This technique also enables analysis of large spaces, like bicycles, cars, rooms, homes.

Molecular data (LC-MS)

Figure 15. Representation of the molecular profile of 2 human volunteers, using 3D-surface-IMS. Molecular diversity is displayed for each volunteer, highlighting regions of high and low diversity on the skin surface. For the color scale, blue corresponds to the minimum value and red corresponds to the maximum value. On the left, male volunteer and on the right, female volunteer. Abbreviations: 3D-surface-IMS: three-dimensional-surface-imaging mass spectrometry; LC-MS: liquid chromatography-mass spectrometry. Modified from Bouslimani et al., 2015.

3D-surface-IMS produces enormous data sets of *m/z* features. These are originated from different samples types with diverse chemicals and each sample type is going to have its own set of molecules. Therefore, the analysis of these big data sets needs an adequate bioinformatics support. Many useful metabolomic analysis tools have been reported in the last 5 years, including MetaMapp (Barupal et al., 2012), MetaMapR (Grapov et al., 2015), Interactive XCMS Online (Gowda et al., 2014) and Warpgroup (Mahieu et al., 2015). Described metabolomic tools are very useful to compare cohorts in clinical data. However, the processing of 3D-surface-IMS data is too complex to apply those tools. Molecular networking strategy offers visualization of chemistries detected by MS on a spectrum by spectrum basis (Watrous et al., 2012). Molecular networking has been implemented at the University of California, San Diego, as a crowd sources analysis infrastructure called Global Natural Product Social molecular networking (GNPS) (Wang et al., 2016), at http://gnps.ucsd.edu. GNPS provides a visualization of MS/MS spectra, their annotation and measurement of their relative similarity to produce a map of all chemicals detected in a single experiment or across a large number of experiments. Thus, GNPS is excellent infrastructure for 3D-surface-IMS data analysis and has been applied in the present article.

In the present work we reported an original article entitled: “Mass Spectrometry-Based Visualization of Molecules Associated with Human Habitats” (Petras et al., 2016). Present study included 5 different human habitats: 2 bicycles, a social event gathering microenvironment, a car, an apartment and a water drinking fountain in a research building and 19 human subjects associated with these environments. The chemistries detected by MS were visualized through multivariate statistical analysis (molecular network) and by 3D cartography. Thanks to a complex analysis, it was possible to assess the chemistry exchange between human and environment and *vice versa*. The original publication is included in the “Original Articles” section.

OBJECTIVES

The overall goal of this work was to develop and validate MS-based analytical methods in order to apply them for basic and clinical research. The specific aims of this thesis were:

1) To develop and validate a MS method for rapid and simple quantification of albendazole and its main metabolite albendazole sulfoxide in human plasma in order to perform pharmacokinetic curves in healthy volunteers and improve treatment efficacy in patients.

2) To improve and validate a MS assay for monitoring of omeprazole plasma levels with the goal of performing a pharmacokinetic analysis and drug-drug interaction analysis in healthy volunteers.

3) To develop a MS method for simultaneous determination of 2 types of neurotransmitters (4 monoamines, 2 amino acids) and 2 metabolites in rat brain tissue and its application in a murine model of depression in order to contribute to the understanding of the neuropathology of depression with the final goal of treatment improvement.

4) To improve the previous method and include 4 different groups of neurotransmitters (14 compounds: monoamines, amino acids, nucleotides and neuropeptides) for their simultaneous determination in bovine chromaffin cells model in order to investigate the neurosecretion profile under stress conditions.

5) To design an untargeted metabolomics IMS approach for visualizations of the chemistry associated with human habitat and its possible future application in clinical medicine, the military, the astrochemistry and to improve forensic research.

Materials and methods as well as results are described in detail in original publications. Citations of these publications and Data in Brief associated to the 3rd publication are listed below:

- Article 1: **Wojnicz, A.**, Cabaleiro-Ocampo, T., Roman-Martinez, M., Ochoa-Mazarro, D., Abad-Santos, F., and Ruiz-Nuno, A. (2013): **A simple assay for the simultaneous determination of human plasma albendazole and albendazole sulfoxide levels by high performance liquid chromatography in tandem mass spectrometry with solid-phase extraction**. *Clin Chim Acta* 426, 58-63.

- Article 2: **Wojnicz, A.**, Gil Garcia, A. I., Roman-Martinez, M., Ochoa-Mazarro, D., Abad-Santos, F., and Ruiz-Nuno, A. (2015): **Improvement and validation of a high-performance liquid chromatography in tandem mass spectrometry method for monitoring of omeprazole in plasma**. *Ther Drug Monit* 37, 381-8.

- Article 3: **Wojnicz, A.**, Avendano Ortiz, J., Casas, A. I., Freitas, A. E., G. Lopez, M., and Ruiz-Nuno, A. (2016b): **Simultaneous determination of 8 neurotransmitters and their metabolite levels in rat brain using liquid chromatography in tandem with mass spectrometry: Application to the murine Nrf2 model of depression**. *Clin Chim Acta* 453, 174-181.

- DiB to article 3: **Wojnicz, A.**, Ortiz, J. A., Casas, A. I., Freitas, A. E., Lopez, M. G., and Ruiz-Nuno, A. (2016c): **Data supporting the rat brain sample preparation and validation assays for simultaneous determination of 8 neurotransmitters and their metabolites using liquid chromatography-tandem mass spectrometry**. *Data Brief* 7, 714-20.

- Article 4: **Wojnicz, A.**, Avendaño-Ortiz, J., de Pascual, R., Ruiz-Pascual, L., García, A. G., and Ruiz-Nuño, A. C. J. M. S. R. (2016a): **Simultaneous monitoring of monoamines, amino acids, nucleotides and neuropeptides by liquid chromatography-tandem mass spectrometry and its application to neurosecretion in bovine chromaffin cells**. *J Mass Spectrom* 51, 651-664.

- Article 5: Petras, D., Nothias, L. F., Quinn, R. A., Alexandrov, T., Bandeira, N., Bouslimani, A., Castro-Falcon, G., Chen, L., Dang, T., Floros, D. J., Hook, V. Y., Garg, N., Hoffner, N., Jiang, Y., Kapono, C. A., Koester, I., Knight, R., Leber, C. A., Ling, T., Luzzatto-Knaan, T., McCall, L. I., McGrath, A. P., Meehan, M. J.,

Merritt, J. K., Mills, R. H., Morton, J., Podvin, S., Protsyuk, I., Purdy, T., Satterfield, K., Searles, S., Shah, S., Shires, S., Steffen, D., White, M., Todoric, J., Tuttle, R., **Wojnicz, A.**, Sapp, V., Vargas, F., Yang, J., Zhang, C., and Dorrestein, P. C. (2016): **Mass Spectrometry-Based Visualization of Molecules Associated with Human Habitats**. *Anal Chem*. DOI: 10.1021/acs.analchem.6b03456

ARTICLE 1

Article 1: A simple assay for the simultaneous determination of human plasma albendazole and albendazole sulfoxide levels by high performance liquid chromatography in tandem mass spectrometry with solid-phase extraction.

Authors: **Aneta Wojnicz**, Teresa Cabaleiro-Ocampo, Manuel Román-Martínez, Dolores Ochoa-Mazarro, Francisco Abad-Santos, Ana Ruiz-Nuño

Clinica Chimica Acta 426 (2013) 58-63

Abstract

A simple, reproducible and fast (4 min chromatogram) method of liquid chromatography in tandem with mass spectrometry (LC-MS/MS) was developed to determine simultaneously the plasma levels of albendazole (ABZ) and its metabolite albendazole sulfoxide (ABZOX) for pharmacokinetic and clinical analysis. Each plasma sample was extracted by solid phase extraction (SPE) using phenacetin as internal standard (IS). The extracted sample was eluted with a Zorbax XDB-CN column using an isocratic method. The mobile phase consisting of water with 1% acetic acid (40%, A) and MeOH (60%, B), was used at a flow rate of 1 mL/min. ABZ and ABZOX were detected and identified by mass spectrometry with electrospray ionization (ESI) in the positive ion and MRM mode. The method was linear in the range of 5-1000 ng/mL for ABZ and 10-1500 ng/mL (full validation) or 10-5000 ng/mL (partial validation) for ABZOX, with 5 and 10 ng/mL lower limit of quantification (LLOQ) for ABZ and ABZOX, respectively. The tests of accuracy and precision, matrix effect, extraction recovery and stability of the samples for both ABZ and ABZOX did not deviate more than 20% for the LLOQ and no more than 15% for other quality controls (QCs), according to regulatory agencies.

Personal contribution: I participated in the conception and design of the study. I contributed in the method development and validation as well as in data generation and analysis. I also contributed to drafting of the paper.

Article 2: Improvement and Validation of a High-Performance Liquid Chromatography in Tandem Mass Spectrometry Method for Monitoring of Omeprazole in Plasma.

Authors: **Aneta Wojnicz**, Ana Isabel Gil García, Manuel Román-Martínez, Dolores Ochoa-Mazarro, Francisco Abad-Santos, and Ana Ruiz-Nuño

Ther Drug Monit 37 (2015) 381-388

Abstract

Background: Omeprazole (OME) is a proton pump inhibitor with a 58% bioavailability after a single oral dose. It is subject to marked interindividual variations and significant drug-drug interactions. The authors developed a simple and rapid method based on liquid chromatography in tandem with mass spectrometry with solid phase extraction and isotope-labeled internal standard to monitor plasma levels of OME in pharmacokinetics and drug-drug interaction studies.

Methods: OME and its internal standard (OME-D3) were eluted with a Zorbax Extend C18 rapid resolution column (4.6 x 50 mm, 3.5 µm) at 25 ºC, under isocratic conditions through a mobile phase consisting of 1 mM ammonium acetate, pH 8.5 (55%), and acetonitrile (45%). The flow rate was 0.8 mL/min, and the chromatogram run time was 1.2 minutes. OME was detected and quantified by liquid chromatography in tandem with mass spectrometry with positive electrospray ionization, which operates in multiple-reaction monitoring mode.

Results: The method was linear in the range of 1.5-2000 ng/mL for OME. The validation assays for accuracy and precision, matrix effect, extraction recovery, and stability of the samples for OME did not deviate more than 20% for the lower limit of quantification and no more than 15% for other quality controls.

Conclusions: These findings are consistent with the requirements of regulatory agencies. The method enables rapid quantification of OME concentrations and can be used in pharmacokinetic and drug-drug interaction studies.

Personal contribution: I participated in the conception and design of the study. I contributed in the method development and validation as well as in data generation and analysis. I also contributed to drafting of the paper.

Article 3: Simultaneous determination of 8 neurotransmitters and their metabolite levels in rat brain using liquid chromatography in tandem with mass spectrometry: Application to the murine Nrf2 model of depression.

Authors: **Aneta Wojnicz** [1], José Avendaño Ortiz [1], Ana I. Casas, Andiara E. Freitas, Manuela G. López, Ana Ruiz-Nuño

[1] Equal contributors

Clinica Chimica Acta 453 (2016) 174-181

Abstract

Analysis of neurotransmitters and their metabolites is useful for the diagnosis of CNS diseases. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with protein precipitation was developed to monitor levels of adrenaline (AD), noradrenaline (NA), glutamic acid (Glu), γ-aminobutyric acid (GABA), dopamine (DA), 5-hydroxytryptamine (5-HT), 5-hydroxyindole acetic acid (5-HIAA), and 3-methoxy-4-hydroxyphenylglycol (MHPG) in rat brain tissue. Isoprenaline was used as an internal standard (IS). Neurotransmitters and metabolites were eluted with a reverse phase column under gradient conditions through a mobile phase consisting of 0.2% formic acid water solution/acetonitrile. The compounds were detected and quantified by LC–MS/MS with positive or negative electrospray ionization, which operates in multiple-reaction monitoring mode. The method was linear or polynomial ($R^2 > 0.99$) for AD, NA, Glu, GABA, DA, 5-HT, 5-HIAA, and MHPG in the range of 0.25-200, 0.5-200, 250-20,000, 250-20,000, 0.25-200, 10-3000, 1-50, and 1-50 ng/mL, respectively. The validation assays for accuracy and precision, matrix effect, extraction recovery, stability and carry-over of the samples for neurotransmitters and metabolites were consistent with the requirements of regulatory agencies. The method enables rapid quantification of neurotransmitters and their metabolites and has been applied in the nuclear factor (erythroid 2-derived)-like 2 (Nrf2) knockout mouse model of depression.

Personal contribution: I participated in the conception and design of the study. I contributed in the method development and validation as well as in data generation and analysis. I also contributed to drafting of the paper.

DiB to article 3: Data supporting the rat brain sample preparation and validation assays for simultaneous determination of 8 neurotransmitters and their metabolites using liquid chromatography-tandem mass spectrometry.

Authors: **Aneta Wojnicz** [1], José Avendaño Ortiz [1], Ana I. Casas, Andiara E. Freitas, Manuela G. López, Ana Ruiz-Nuño

[1] Equal contributors

Data in Brief 7 (2016) 714-720

Abstract

The data presented in this article supports the rat brain sample preparation procedure previous to its injection into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system to monitor levels of adrenaline, noradrenaline, glutamicacid, γ-aminobutyric acid, dopamine, 5-hydroxytryptamine, 5-hydroxyindoleaceticacid, and 3-methoxy-4-hydroxyphenylglycol. In addition, we describe the method validation assays (such as calibration curve, lower limit of quantification,precisionandaccuracyintra-andinter-day, selectivity, extraction recovery and matrix effect, stability, and carry-over effect) according to the United States Food and Drug Administration and European Medicine Agency to measure in one step different neurotransmitters and their metabolites. The data supplied in this article is related to the research study entitled: “Simultaneous determination of 8 neurotransmitters and their metabolite levels in rat brain using liquid chromatography in tandem with mass spectrometry: application to the murine Nrf2 model of depression” (Wojnicz et al., 2016b)

Personal contribution: I participated in the conception and design of the study. I contributed in the method development and validation as well as in data generation and analysis. I also contributed to drafting of the paper.

Article 4: Simultaneous monitoring of monoamines, amino acids, nucleotides and neuropeptides by liquid chromatography-tandem mass spectrometry and its application to neurosecretion in bovine chromaffin cells.

Authors: **Aneta Wojnicz**, José Avendaño-Ortiz, Ricardo de Pascual, Lucía Ruiz-Pascual, Antonio G. García, and Ana Ruiz-Nuño

J. Mass Spectrom 51 (2016) 651-664

Abstract

The primary functions of adrenal medullary chromaffin cells are the synthesis and storage in their chromaffin vesicles of the catecholamines noradrenaline (NA) and adrenaline (AD), and their subsequent release into the bloodstream by Ca^{2+}-dependent exocytosis under conditions of fear or stress (fight or flight response). Several monoamines, nucleotides and opiates, such as leucine-enkephalin (LENK) and methionine-enkephalin (MENK), are also co-stored and co-released with the catecholamines. However, other neurotransmitters have not been studied in depth. Here, we present a novel high-resolution liquid chromatography-tandem mass spectrometry approach for the simultaneous monitoring of 14 compounds stored and released in BCCs. We validated the analytical method according to the recommendations of the EMA and FDA by testing matrix effect, selectivity, sensitivity, precision, accuracy, stability and carry-over. After testing on six batches of BCCs from different cultures, the method enabled simultaneous quantitative determination of monoamines (AD, NA, dopamine, serotonin, 5-hydroxyindoleacetic acid, histamine and metanephrine), amino acids (L-glutamic acid, γ-aminobutyric acid), nucleotides (adenosine 5′-diphosphate, adenosine 5′-monophosphate, cyclic adenosine 5′-monophosphate) and neuropeptides (LENK and MENK) in the intracellular content, basal secretion and acetylcholine induced secretion of BBCs. The high-resolution approach used here enabled us to determine the levels of 14 compounds in the same BCC batch in only 16 min. This novel approach will make it possible to study the regulatory mechanisms of Ca^{2+} signaling, exocytosis and endocytosis using different neurotrophic factors and/or secretagogues as stimuli in primary BCC cultures. Our method is actually being applied to human plasma samples of different therapeutic areas where sympathoadrenal axis is involved in stress situations such as Alzheimer's disease, migraine or cirrhosis, to improve diagnosis and clinical practice.

Personal contribution: I participated in the conception and design of the study. I participated in the method development and validation as well as in data generation and analysis. I also participated in the drafting of the paper.

Article 5: Mass Spectrometry-Based Visualization of Molecules Associated with Human Habitats.

Authors:

Daniel Petras, Louis-Félix Nothias, Robert A. Quinn, Theodore Alexandrov, Nuno Bandeira, Amina Bouslimani, Gabriel Castro-Falcón, Liangyu Chen, Tam Dang, Dimitrios J. Floros, Vivian Hook, Neha Garg,¶ Nicole Hoffner, Yike Jiang, Clifford A. Kapono, Irina Koester, Rob Knight, Christopher A. Leber, Tie-Jun Ling,¶Tal Luzzatto-Knaan,¶ Laura-Isobel McCall, Aaron P. McGrath, Michael J. Meehan,¶Jonathan K. Merritt, Robert H. Mills, Jamie Morton, Sonia Podvin, Ivan Protsyuk, Trevor Purdy, Kendall Satterfield, Stephen Searles, Sahil Shah, Sarah Shires, Dana Steffen, Margot White, Jelena Todoric, Robert Tuttle, **Aneta Wojnicz**,¶ Valerie Sapp, Fernando Vargas, Jin Yang, Chao Zhang and Pieter C. Dorrestein

Anal Chem: DOI: 10.1021/acs.analchem.6b03456

Abstract

The cars we drive, the homes we live in, the restaurants we visit, and the laboratories and offices we work in are all a part of the modern human habitat. Remarkably, little is known about the diversity of chemicals present in these environments and to what degree molecules from our bodies influence the built environment that surrounds us and vice versa. We therefore set out to visualize the chemical diversity of five built human habitats together with their occupants, to provide a snapshot of the various molecules to which humans are exposed on a daily basis. The molecular inventory was obtained through untargeted liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of samples from each human habitat and from the people that occupy those habitats. Mapping MS-derived data onto 3D models of the environments showed that frequently touched surfaces, such as handles (e.g., door, bicycle), resemble the molecular fingerprint of the human skin more closely than other surfaces that are less frequently in direct contact with humans (e.g., wall, bicycle frame). Approximately 50% of the MS/MS spectra detected were shared between people and the environment. Personal care products, plasticizers, cleaning supplies, food, food additives, and even medications that were found to be a part of the human habitat. The annotations indicate that significant transfer of chemicals takes place between us and our built environment. The workflows applied here will lay the foundation for future studies of molecular distributions in medical, forensic, architectural, space exploration, and environmental applications.

Personal contribution: I assisted the “System Wide Mass Spectrometry” course held by Professor Pieter Dorrestein, at University of California, San Diego during my short term stay in the USA, where I helped with samples collection, preparation and data generation.

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DISCUSSION

Discussion

MS is nowadays a state-of-art clinical tool, a robust and indispensable research instrument able to “make fly molecular elephants” and therefore it allows for a wide range of applications. In fact, there is no other device that has contributed to so many fields over the past 100 years (Bowers, 1989; Fenn, 2003; Maher et al., 2015). In our laboratory, MS is used on a daily basis, both for basic and clinical research. It helped us to perform pharmacokinetic studies of ABZ, ABZOX (Wojnicz et al., 2013) and OME (Wojnicz et al., 2015) in order to improve treatment of patients, and in the future these studies could lead to personalized medicine development. We used MS for NTs research (Wojnicz et al., 2016a; Wojnicz et al., 2016b) to better understand neuropsychiatric and neurodegenerative diseases, such as major depressive disorder or Alzheimer’s disease with the aim to improve diagnosis and treatment. During my short stay at University of California, San Diego, I have taken an active part in another important application of MS, this time the understanding of chemistry transfer between humans and their environment and *vice versa* (Petras et al., 2016). This last study could have an impact on more conscientious and healthy human environments design and also improve forensic research.

Four methods presented in this work (articles 1 to 4 and DiB) were validated according to FDA (FDA, 2001) and EMA (EMA, 2011) guidelines on bioanalytical method validation, including the following tests: selectivity and linearity, calibration curve, precision and accuracy, recovery, matrix effect, stabilities and, in some cases, carry over. In this thesis, we are not going to discuss the precision, accuracy and stability tests, since all methods presented here meet FDA and EMA requirements. However, we will discuss the following tests: MS/MS conditions optimization, selectivity and sensitivity, sample preparation procedure followed by recovery and matrix effect, sample volume and time of chromatographical run, carry-over effect, and finally the application of the validated method. The selected tests are very variable between methods, thus it is essential to discuss how they can influence the final results. The 5th method represented an untargeted metabolomics method and different strategies for method validation were applied. In the case of the 5th method, sample preparation and MS data processing and the final application, will be discussed.

1. Application of LC-MS for clinical pharmacokinetic studies

1.1. Albendazole and albendazole sulfoxide (Article 1)

Due to the poor absorption of ABZ and differences of hydrophobic character between ABZ and ABZOX, ABZ is rapidly metabolized to its primary active metabolite ABZOX. This causes that plasma levels of ABZ are very low and it makes it difficult to obtain a good pharmacokinetic curve. The half-life of ABZOX ranges from 8 to 12 hours (single dose of ABZ, 400 mg/d). For this reason, ABZOX is often chosen as the only compound for pharmacokinetic determinations (Saraner et al., 2016). However, in this work, highly sensitive and reproducible LC-MS/MS method was optimized and validated for simultaneous quantification of both ABZ and ABZOX in human plasma for pharmacokinetic studies and clinical practice to improve the effectiveness of treatment (Wojnicz et al., 2013).

Table 3 shows the overview of methods applied for determination of ABZ and its metabolites in body fluids up to now. Methods are listed in the table depending on the technique applied and the year of publication, from the earliest to the most recent. The table contains 12 published methods: 3 HPLC-UV, 1 HPLC-FLD, 1 HPLC-UV/FLD, 1 HPLC-NACE (HPLC-non-aqueous capillary electrophoresis) and 6 LC-MS/MS methods. The parameters of published methods from the left to the right include: analyte(s) analysed and LLOQ achieved, sample type, volume and extraction procedure, IS applied, chromatographic separation type and time of analysis as well as injection volume (INY) for single sample, the percentages of recoveries and matrix effect achieved. Finally, the method we developed is highlighted in green in the table. Recoveries are expected to be calculated for all validated methods. However, matrix effect is mandatory only for LC-MS/MS validated approaches. This table highlights important issues of method validation and helps to discuss the results obtained.

1.1.1. Method validation

MS/MS conditions

The mass spectrometry was operated in MRM mode. ESI in positive mode was selected for both ABZ and ABZOX. Although RT of ABZOX (2.126 min) and its IS (2.326 min) are very close, they can be separated by the analysis of extraction ion chromatogram based on reconstructed ion currents.

Method	Analyte(s); LLOQ (ng/mL)	Sample volume; type; extraction procedure	IS	Separation type; time; INY (µL)	Recovery; Matrix effect (%)	Author
HPLC-UV	ABZ, ABZOX; 30	2 mL; plasma, CSF; SPE	mebendazole	Isocratic; NI; NI	R: 95-100	(Hurtado et al., 1989)
	ABZ, ABZOX (+1 more); 20	1 mL; plasma; SPE	oxibendazole	Isocratic; 30 min; 5 ,10	R: 95-98	(Kitzman et al., 2002)
	ABZ, ABZOX (+1 more); 50, 25	1 mL; plasma; PPT	mebendazole	Isocratic; 15 min; 10-50	R: 79-93	(Sarin et al., 2004)
HPLC-FLD	ABZOX enatiomers (+1 more); 5	0.5 mL; plasma; LLE	NI	Isocratic; NI; NI	NI	(Lanchote et al., 1998)
HPLC-UV/FLD	ABZ, ABZOX (+1 more); NI	NI; serum; LLE	mebendazole	Isocratic; 10 min; 100	R: 65-96	(Mirfazaelian et al., 2002)
HPLC-NACE	ABZ, ABZOX (+1 more); NI	0.5 mL; plasma; LLE	mebendazole	Isocratic; 8 min; NI	R: 63-98	(Prochazkova et al., 2000)
LC-MS/MS	ABZOX (+1 more); 5	1 mL; plasma; LLE	phenacetin	Isocratic; 15 min; 50	R: ≥85 ; ME: NI	(Bonato et al., 2003)
	ABZ, ABZOX; 0.4 and 4	0.5 mL; plasma; LLE	estazolam	Isocratic; 5 min; 20	R: 53-77; ME: NI	(Chen et al., 2004)
	ABZOX (+1 more); 5	1 mL; plasma; LLE	phenacetin	Isocratic; 10 min; 50	R: 64; ME: NI	(Bonato et al., 2007)
	ABZOX (+1 more); 20	0.2 mL; plasma, CSF; LLE	carbamezapine	Isocratic; <5 min; 25	R ≥90; ME: 80-120	(Gonzalez-Hernandez et al., 2012)
	ABZ, ABZOX; 5, 10	**0.2 mL; plasma; SPE**	**phenacetin**	**Isocratic; 4 min; 5**	**R: 93-109; ME: 93-99**	***(Wojnicz et al., 2013)**
	ABZOX; 3	0.2 mL; plasma; PPT	ABZOX-D_3	Isocratic; 3.5 min; 5	R: 103; ME: 80-120	(Saraner et al., 2016)

Table 3. Overview of methods applied for determination ABZ and its metabolites in body fluids in comparison with the literature. Abbreviations: ABZ: albendazole; ABZOX: albendazole sulfoxide; ABZOX-D_3: isotope labeled albendazole sulfoxide; CSF: cerebrospinal fluid; HPLC-UV, high-performance liquid chromatography-ultraviolet detection; HPLC-FLD, high-performance liquid chromatography-fluorescence detection; HPLC-UV/FLD, high-performance liquid chromatography-ultraviolet/fluorescence detection; HPLC-NACE, high-performance liquid chromatography-non-aqueous capillary electrophoresis; INY: injection volume; IS: internal standard; LLE: liquid-liquid extraction; LLOQ: lower limit of quantification; LC-MS/MS, liquid chromatography-tandem mass spectrometry; ME: matrix effect; NI: not informed; PPT: protein precipitation; R: extraction recovery; SPE: solid phase extraction. *The results obtained with the method we developed are highlighted in green.

Method selectivity and sensitivity

Good sensitivity of an analytical method is one of the most important factors to take into account when analytical method is being developed. It depends on the instrument, but also on the sample extraction procedure applied. To date, the lowest LLOQs were obtained by Chen and co-workers with the value of 0.4 ng/mL for ABZ (Chen et al., 2004) and by the Saraner group with the value of 3 ng/mL for ABZOX (Saraner et al., 2016). In our method, the LLOQs were as follows: 5 ng/mL for ABZ and 10 ng/mL for ABZOX (Wojnicz et al., 2013). The particle size of the column (3.5 µm) used by Saraner and colleagues was significantly smaller than of the column (5 µm) we used and could underlie the differences observed in LLOQs. The column particle size has an important influence on the LLOQs, in general the smaller the particle size, the better LLOQs. While not reaching the before-mentioned values, we still outperformed other methods developed by different authors (Gonzalez-Hernandez et al., 2012; Hurtado et al., 1989; Kitzman et al., 2002; Sarin et al., 2004).

Sample extraction procedure, recoveries and matrix effect

The choice of the sample extraction procedure should depend on the method of detection applied. In the case of HPLC-UV, HPLC-FLD or HPLC-non-aqueous CE (HPLC-NACE), the matrix effect test is not an issue. The optimal range of recovery is within 80-120%. However, when the LC-MS/MS method with ESI is applied, we should take into account factors, which can modify desolvatation and ionization process of the sample (e.g. peptides, endogenous phospholipids, mobile phase modifiers and formulation agents) (Chambers et al., 2007; Larger et al., 2005), thus producing the loss of the analyte signal (King et al., 2000).

As seen in the Table 3, the mean recoveries of the previously applied techniques were between 79-93% (Sarin et al., 2004), except for Saraner and co-workers with a very good result of 103%. The latter is likely caused by the use of isotope-labeled IS in the PPT process (Saraner et al., 2016). The results obtained using LLE were ranging within 53-98% (Bonato et al., 2007; Bonato et al., 2003; Chen et al., 2004; Gonzalez-Hernandez et al., 2012; Lanchote et al., 1998; Mirfazaelian et al., 2002; Prochazkova et al., 2000). It seems that SPE effectively eliminated matrix interferences as the best values of recoveries without IS were obtained with SPE in the range of 93-100% (Hurtado et al., 1989; Kitzman et al., 2002; Wojnicz et al., 2013). Even though matrix effects were not evaluated in 3 of 6 LC-MS/MS described methods (Bonato et al., 2007; Bonato et al., 2003; Chen et al., 2004), the values of matrix effect

in our work are similar to other methods (Gonzalez-Hernandez et al., 2012; Saraner et al., 2016; Wojnicz et al., 2013) and meet the requirements of regulatory agencies (EMA, 2011; FDA, 2001).

Sample volume

Sample volume used for the extraction procedures is also a very important issue to consider in the analytical method optimization. It must be reduced due to the stress produced in patients during blood extraction and the total amount of blank plasma needed for bioanalytical method development and validation (a minimum of 50 mL of plasma is needed). Many protocols described in the literature (Table 3) used large volumes of plasma, from 2 mL (Hurtado et al., 1989), 1 mL (Bonato et al., 2007; Bonato et al., 2003; Kitzman et al., 2002; Sarin et al., 2004) to 0.5 mL (Chen et al., 2004; Lanchote et al., 1998; Prochazkova et al., 2000). In our extraction method, we have significantly reduced the volume of plasma needed to only 0.2 mL (Wojnicz et al., 2013), thus reaching the lowest volume requirements documented in the literature (Gonzalez-Hernandez et al., 2012; Saraner et al., 2016).

Chromatographic separation, time of analysis and sample injection volume

Due to the differences in water solubility of ABZ and its metabolite, the chromatographic separation in all described methods was isocratic. However, the time of analysis of a single sample is another important factor to consider. Its importance increases especially when large number of samples for clinical pharmacokinetic studies is processed. Methods published in the literature presented analysis times between 8-15 min (Bonato et al., 2007; Bonato et al., 2003; Mirfazaelian et al., 2002; Prochazkova et al., 2000; Sarin et al., 2004), or even 30 min (Kitzman et al., 2002). The more “attractive” assays optimized the analysis times to 5 min (Chen et al., 2004; Gonzalez-Hernandez et al., 2012). In the method described in this thesis, the LC-MS/MS instrument spent only 4 min for sample acquisition (Wojnicz et al., 2013). There is only one method requiring even shorter analysis time (3.5 minutes), published recently by Saraner and co-workers (Saraner et al., 2016). That can be explained by the use of a “superior” column (100 mm long and 3.5 µm particle size) than ours (150 mm long and 5 µm particle size).

Moreover, taking into account the particle size of the column, we have followed the manufacturer’s recommendations and injected 5 µL of the sample into the LC-MS/MS system. However, Saraner and co-workers have used similar injection volume, even when the column particle size was smaller (3.5 µm) than in our case (5 µm). On

the other hand, other authors have used a larger injection volumes for LC-MS/MS analysis, around 20 µL and even 50 µL (Bonato et al., 2007; Chen et al., 2004), which had a negative effect on analyte recoveries.

Carry-over

Although carry-over effect evaluation is not mandatory in all guidelines for bioanalytical method validation, it should be assessed within the method development and validation. Determination of carry-over effect (by injecting blank solvent solution after standard calibrator with the highest concentration) is a very simple way to detect a possible instrument contamination and can be included routinely in each analytical run. Carry-over effect was not evaluated in the Publication 1 concerning the ABZ and ABZOX method, similarly to other authors (Bonato et al., 2003; Saraner et al., 2016). However, this test was performed after assay publication and no significant carry-over effect was observed, confirming no need for concerns about column contamination when performing this method (data not shown).

1.1.2. Application of albendazole and albendazole sulfoxide method

Considering our results, we have achieved C_{max} of 57.5 +/- 52.3 ng/mL for ABZ and 830 +/- 440 ng/mL for ABZOX in healthy volunteers (Wojnicz et al., 2013). Similar results in healthy volunteers were obtained by Chen and co-workers with C_{max} values 96.4 +/- 50.1 ng/mL for ABZ and 635 +/- 229 ng/mL for ABZOX (Chen et al., 2004) and the Kitzman group with C_{max} values of ≈200 ng/mL for ABZ and ≈1000 ng/mL for ABZOX (Kitzman et al., 2002). However, pharmacokinetics of ABZOX seems to vary considerably in neurocystercosis patients, from low C_{max} of 85.3 ng/mL reported by Bonato and co-workers (Bonato et al., 2003) to very high mean plasma concentration of 1991.4 +/- 925.9 ng/mL (Gonzalez-Hernandez et al., 2012). These results confirm the need of TDM of ABZ and ABZOX, due to large interindividual variations of plasma concentration. All validated methods were applied successfully to the ABZ and/or ABZOX plasma monitoring, except of Prochazkova and co-workers (Prochazkova et al., 2000). In this study, the LOD for ABZ was too high, making it impossible to perform pharmacokinetic curves of those compounds in real samples.

1.2. Omeprazole (Article 2)

OME is a proton pump inhibitor used as a stomach protective prodrug, which does not require a medical prescription. For this reason, it is one of the most consumed drugs nowadays However, it is important to highlight that proton pump inhibitors not only

inhibit H^+/K^+-ATPase pumps in the stomach, but also proton pumps of the whole body, including the brain. Actually, it was demonstrated that OME has a significant effect on CSF production in animals (Javaheri et al., 1997; Lindvall-Axelsson et al., 1992). Taking into account the existence of interindividual genetic differences in *CYP* (Clissold and Campoli-Richards, 1986; Macek et al., 2007; Oh et al., 2012) and possible drug-drug interactions leading to the pharmacokinetic variability, TDM of OME is required. It is especially important for patients suffering from chronic diseases, who are often polymedicated and/or using drugs with a narrow therapeutic index (Robinson and Horn, 2003; Wedemeyer and Blume, 2014). For these reasons, we have developed and validated a simple and sensitive LC-MS/MS approach to determine plasma levels of OME to carry out pharmacokinetic studies and to improve clinical practice.

Table 4 displays a review of published methods describing OME and/or their metabolites determination in human plasma. Methods are listed in the table depending on the technique applied and the year of publication, from the earliest to the most recent. The table contains 19 published methods: 9 HPLC-UV, 1 CE-UV, 1 HILIC-MS/MS and 8 LC-MS/MS methods. The parameters of published methods from the left to the right include analyte(s) analysed and LLOQ achieved, sample type, volume and extraction procedure, IS applied, chromatographic separation type, time of analysis and INY for single sample, the percentages of recoveries and matrix effect achieved. This table uncovers important issues of method validation and helps to discuss the results. In the table, the method we have developed is highlighted in green.

Method	Analyte(s); LLOQ (ng/mL)	Sample volume; type; extraction procedure	IS	Separation type; time; INY (µL)	Recovery; Matrix effect (%)	Author
HPLC-UV	OME; 5	0.27 mL; plasma; SPE	phenacetin	Isocratic; 25 min; 100	R: 85	(Garcia-Encina et al., 1999)
	OME; 5	1 mL; plasma; SPE	chloramphenicol	Isocratic; 10 min; 20	R: 96	(Yuen et al., 2001)
	OME (+2 more); 96	0.5 mL; plasma; LLE	phenacetin	Isocratic; 10 min; 100	R: 99	(Gonzalez et al., 2002)
	OME (+2 more); 3	1 mL; plasma; LLE	lansoprazole	Isocratic; ≈25 min; 30	R: 85	(Shimizu et al., 2006)
	OME; 10	NI; plasma; NI	NI	Isocratic; no data; NI	R: ≤100	(Zarghi et al., 2006)
	OME (+3 more); 20.61	0.5 mL; plasma; LLE	zonisamide	Isocratic; 11 min; 20	R: 67	(Bharathi et al., 2009)
	OME (+3 more); 20	1 mL; plasma; LLE	pantoprazole lansoprazole	Isocratic; 10 min; 50	R: 84	(Noubarani et al., 2010)
	OME (+2 more); 5	1 mL; plasma; LLE	lansoprazole	Gradient; 8.2-50 min; 30	R: 76	(Shiohira et al., 2011)
	OME (+2 more); 50	1 mL; plasma; LLE	NI	Gradient; 10 min; 20	R: 97	(Ahmed and Atia, 2015)
CE-UV	OME (+2 more); 80	1 mL; plasma; automated SPE	NI	NI; 6.5 min; 100	R: 99	(Perez-Ruiz et al., 2006)
HILIC-MS/MS	OME (+1 more); 2.5	0.05 mL; plasma; automated LLE	desoxyomeprazole	Gradient; 2.75 min; 10	R: 93	(Song and Naidong, 2006)
LC-MS/MS	OME (+1 more); 10	NI; plasma; SPE	NI	Isocratic; 2.75 min; NI	NI	(Woolf and Matuszewski, 1998)
	OME (+3 more); 0.4	0.25 mL; plasma; LLE	flunitrazepam	Isocratic; 15 min; 20	R: 91; ME: NI	(Frerichs et al., 2005)
	OME;1.2	0.25 mL; plasma; PPT	flunitrazepam	Isocratic; 1.35 min; 1	NI	(Macek et al., 2007)
	OME; 0.05	0.3 mL; plasma; LLE	lansoprazole	Isocratic; 2.5 min; 10	R: 91; ME: 80-120	(Vittal et al., 2009)
	OME (+4 more); 1.145	0.1 mL; plasma; on-line SPE;	imipramine	Isocratic; 6 min; 5	R: ≥85; ME: NI	(Dodgen et al., 2011)
	OME (+9 more); 0.05	0.45 mL; plasma; LLE	propanolol	Isocratic; 10 min; 5	R: ≥63 ME: NI	(Oh et al., 2012)
	OME; 1.5	**0.2 mL; plasma; SPE**	**OME-D3**	**Isocratic; 1.2 min; 1**	**R: 95; ME: 98**	***(Wojnicz et al., 2015)**
	OME; 4	0.1 mL; plasma; automated LLE	pantoprazole	Isocratic; 1.5 min; 30	R: 85; ME: 98	(Koukoula et al., 2016)

Table 4. **Overview of methods applied for determination OME and its metabolites in body fluids in the literature.** Abbreviations: CE-UV: capillary electrophoresis-ultraviolet detection; HILIC-MS: hydrophilic interaction chromatography-mass spectrometry; HPLC-UV, high performance liquid chromatography-ultraviolet detection; INY: injection volume; IS: internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLE: liquid-liquid extraction; LLOQ: lower limit of quantification; ME: matrix effect; NI: not informed; OME: omeprazole; OME-D3: isotope labeled OME, IS; PPT: protein precipitation; R: extraction recovery; SPE: solid phase extraction. *The results obtained with the method we developed are highlighted in green.

1.2.1. Method validation

MS/MS conditions

The MS was operated in MRM mode. ESI in positive mode was selected for OME. When only 1 compound is included in LC-MS/MS method, there is generally no difficulty for targeted MS/MS optimization, thus we are not going to discuss this part of the method development.

Method selectivity and sensitivity

Bioanalytical method presented in this work offers a wide calibration range (1.5-2000 ng/mL) for OME plasma quantification. Thus, it can be applied even for high OME plasma levels, which can be expected due to intersubject's variability. In our assay, the LLOQ was set at 1.5 ng/mL. Although more sensitive LC-MS/MS methods have been reported, with LLOQs of 1.2 (Macek et al., 2007), 1.145 (Dodgen et al., 2011), 0.4 (Frerichs et al., 2005) or even 0.05 ng/mL (Oh et al., 2012; Vittal et al., 2009), most of these methods have used greater plasma volumes for extraction than we have, except of Dodgen and co-workers, who have used automated on-line LLE with significant plasma volume reduction. Of note, all LC-MS/MS based assays, including ours, provide better selectivity and sensitivity, than those using UV detection characterized by higher LLOQ values between 3 and 96 ng/mL (Ahmed and Atia, 2015; Bharathi et al., 2009; Garcia-Encina et al., 1999; Gonzalez et al., 2002; Noubarani et al., 2010; Shimizu et al., 2006; Shiohira et al., 2011; Yuen et al., 2001; Zarghi et al., 2006); or coulometric detection, with LLOQ of 6 ng/mL (Sluggett et al., 2001); and also CE methods with UV or MS detection with LLOQs of 80 (Perez-Ruiz et al., 2006) and 35 ng/mL (Nevado et al., 2014), respectively.

Sample volume

Sample volume varied greatly between listed methods, from 1 mL (Ahmed and Atia, 2015; Noubarani et al., 2010; Shimizu et al., 2006; Shiohira et al., 2011; Yuen et al., 2001), 0.45-0.5 mL (Bharathi et al., 2009; Gonzalez et al., 2002; Oh et al., 2012), 0.20-0.25 mL (Frerichs et al., 2005; Macek et al., 2007; Wojnicz et al., 2015) to 0.1 mL (Dodgen et al., 2011; Koukoula et al., 2016), or even 0.05 mL (Song and Naidong, 2006). We have used plasma volume of 0.2 mL, 2 to 4 times more than the smallest sample volume described (Dodgen et al., 2011; Koukoula et al., 2016; Song and Naidong, 2006). This is caused by differences in the extraction method; our SPE procedure was manual and required larger sample volumes as compared to the automated on-line LLE or SPE used in the other studies (Dodgen et al., 2011;

Koukoula et al., 2016; Song and Naidong, 2006) allowing to reach the smallest volumes.

Chromatographic separation, time of analysis and sample injection volume

For OME chromatographic run presented in our method, we used isocratic separation on Zorbax Extend C18 high-resolution column. This column enables high flow rates (0.8 mL/min) and rapid run times. For this reason, our approach achieves the shortest analysis time of 1.2 min among the methods described in the literature (Wojnicz et al., 2015). Similar results were obtained by Macek and co-workers (Macek et al., 2007), requiring a run time of 1.35 min, followed by Koukoula and co-authors (Koukoula et al., 2016) with 1.5 min as a total run time. When LC-MS/MS with ESI is applied and the particle size of the analytical column is small (up to 3.5 µm), small injection volume is highly recommended to avoid overloading, matrix effect and instrument contamination (Jessome and Volmer, 2006). Thus, we have used very small injection volume of the sample (1 µL). Up to now, there is only 1 method using the same sample injection (Macek et al., 2007). Macek and colleagues have used greater plasma volume (0.25 mL) than us (0.2 mL) to achieve lower LLOQ. Other authors used injection volumes between 5 and 30 µL (Frerichs et al., 2005; Koukoula et al., 2016; Oh et al., 2012; Vittal et al., 2009), even with LC-MS/MS and ESI detection.

Carry-over

Determination of carry-over effect helps importantly to prevent contamination. If detected, optimal washing procedures of the injection needle and the analytical column should be applied to guarantee the precision and accuracy of the method. However, only few authors listed in table 4 have run this test (Dodgen et al., 2011; Koukoula et al., 2016; Macek et al., 2007; Wojnicz et al., 2015). Similarly to others, we have obtained results showing no significant carry-over. Of note, the carry-over effect of our method was lower (<7.5%) than the one detected by Macek and colleagues (<10%).

1.2.2. Application of omeprazole method

Almost all of the mentioned assays have been applied with success for OME plasma determination in order to perform pharmacokinetic studies and/or drug-drug interaction analysis. Our method was used for OME quantification in 6 healthy volunteers (240 samples in total) after oral administration of OME (40 mg/d) under fasting conditions. C_{max} of OME we obtained (1395.18 +/- 814.67 ng/mL) was similar to that achieved by Liu and co-workers (1330.46 +/- 758.07 ng/mL (Liu et al., 2012)), and

slightly higher than that of the Ahmed group (950 +/- 0.17 ng/mL (Ahmed and Atia, 2015)). The results were within the higher C_{max} range in fasting condition compared to fat condition, as described by the Vaz-da-Silva group (Vaz-da-Silva et al., 2005). Although other authors reported lower OME C_{max}, the differences can be explained by lower OME administration dose (Oh et al., 2012; Perez-Ruiz et al., 2006; Shimizu et al., 2006). Moreover, we have also observed a large interindividual variety of OME concentration, which can be explained by different volunteers' genotype (Koukoula et al., 2016; Shimizu et al., 2006). Additionally, 3 of 6 volunteers have been taking concomitant therapy during the clinical trial (norgestimate-ethinyl estradiol, acetaminophen and ibuprofen), and with our assay we were able to achieve a good selectivity, since no significant interference was observed.

The discussed methods concerning ABZ, ABZOX (Article 1) and OME (Article 2) plasma quantification by LC-MS/MS highlight the important role of these kinds of assays in pharmacokinetic studies and TDM in order to improve routine clinical practice. However, as clear from the discussion so far, all the methods show a great variability in plasma levels as measured in different laboratories. Of course genetic background and concomitant therapy play an important role and could cause these differences, together with the method applied for the sample analysis. Each method used different detectors, concentration ranges, sample extraction methods, sample volume etc. Thus, for successful development of personalized medicine, cross-validation of bioanalytical methods should be done between laboratories.

2. Application of LC-MS for neurotransmitters research

Monitoring of NTs and their metabolites from CNS and body fluids provides an interesting field of investigation. Strikingly, according to the new co-transmission theory, many known and unknown NTs are released from a single cell to perform their physiological or pathological effect. For this reason, modern analytical methods focus on developing assays capable of simultaneous determination of as many as possible NTs and their metabolites, and preferably in one single analytical run. However, difficulties may arise due to the different physical-chemical properties of NTs and their metabolites, which are divided into acetylcholine neurotransmitters, monoamines, amino acids, neuropeptides and nucleotides (e.g. monoamine NTs produce mostly acidic metabolites, etc. (Bicker et al., 2013)). Thus, the design of a single bioanalytical method capable of detecting and quantifying all NTs and their metabolites becomes quite complicated. However, it is not impossible and it requires finding optimal conditions to solve the problem.

Table 5 summarizes the overview of some of the methods published in the literature, where different NTs and/or their metabolites were determinated in rat/mouse/cat brain tissue and/or in adrenal medulla/BCCs/other cells and/or vesicles (see the pages 200 and 201). Methods are listed in the table depending on the technique applied and the year of publication, from the earliest to the most recent. Table 5 includes 31 published methods: 1 HPLC-UV, 8 HPLC-ED and 1 UHPLC-ED, 2 HPLC-FLD, 1 RIA, 12 LC-MS/MS and 4 UPLC-MS/MS, 1 liquid chromatography-high resolution mass spectrometry (LC-HRMS) and 1 DESI-IMS. The parameters of published methods from the left to the right include: analyte(s) analysed and LLOQ achieved, sample type and extraction procedure, IS applied, chromatographic separation type and time of analysis for single sample and sample INY volume, the percentages of recoveries and matrix effect achieved. This table brings up important issues of method validation and help to discuss the results obtained. In the table, the methods we developed are highlighted in green.

Method	Analyte(s)	Sample type; extraction procedure	IS	Separation type; time; INY (µL)	Recovery; Matrix effect (%)	Author
HPLC-UV	Glu, GABA (+3 more)	Rat hippocampus; PPT	NI	Isocratic; 15 min; 10	R: 95-105	(Wu et al., 2013)
HPLC-ED	NA, AD, DA	Rat adrenal medullary cells secretion, NT	NI	Isocratic; ≈10 min; NI	NI	(Muller and Unsicker, 1981)
	NA, DA, 5-HT, MHPG, MN, 5-HIAA (+ 2 more)	Rat brain tissue, NI	NI	Isocratic; NI; NI	NI	(Kotake et al., 1985)
	(NA, DA, 5-HT, MHPG, 5-HIAA (+4 more)	Mouse brain tissue, NI	NI	Gradient; 10 min; NI	NI	(Murai et al., 1988)
	NA, AD, DA	Human foetal adrenal gland, PPT	1 IS	Isocratic; ≈28 min; 5-20	NI	(García et al., 1994)
	2 methods: NA, AD, MN, MHPG, DA, MENK (+4 more)	Dog adenal gland perfusate, supernatant, NTT	1 IS	Gradient; 42,2 min; NI	R: 76-83	(Chritton et al., 1997)
	NA, AD, DA, 5-HT, 5-HIAA (+ 2 more)	Cultured nerve cells microdialysis, NTT	NI	Isocratic; 18 min; NI	R: 30-49	(Xu et al., 2002)
	NA, AD, MHPG, DA, 5-HT, 5-HIAA (+ 2 more)	Mouse brain tissue, NI	2 ISs	Isocratic; NI; NI	R: ≈100	(Nguyen et al., 2010)
	NA, DA, 5-HT, 5-HIAA (+ 2 more)	Rat brain tissue, PPT	NI	Isocratic; NI; 20 µL	NI	(Del Pino et al., 2011)
UHPLC-ED	NA, DA, 5-HT, 5-HIAA (+ 2 more)	Rat, mouse, cat brain tissue, PPT	NI	Isocratic; 5 min; 1	NI	(Parrot et al., 2011)
HPLC-FLD	NA, DA, 5-HT, HIST (+ 2 more)	Bovine plasma, urine, tissue; PPT	1 IS	Gradient; 82 min; 10	R: 55-78	(Davis et al., 1978)
	NA, 5-HT, 5-HIAA	Rat brain tissue microdialysis; PPT	NI	Isocratic; 30 min; 20	NI	(Yoshitake et al., 2003)
RIA	NA , AD, DA, MENK (+ 3 more)	BCC vesicles; NT	NI	NA	NI	(Podvin et al., 2015)
LC-MS/MS	AMP, ADP (+ 1 more)	Cultures rat C6 glioma cells secretion; NTT	NI	Gradient; 16 min; NI	NI	(Qian et al., 2004)
	5-HT, 5-HIAA (+ 2 more)	Rat brain tissue; PPT	NI	Gradient; 10 min; 10	NI	(Lang et al., 2004)
	DA, 5-HT (+ metabolites)	Rat brain tissue; SPE	Isotope labeled ISs	Gradient; NI	NI	(Tareke et al., 2007)
	NA, AD, DA, 5-HT	BCCs secretion; NT	NI	Gradient; 6 min; 25	NI	(Carrera et al., 2007)
	2 methods: NA, AD, DA, MN, MHPG (+ 8 more)	Rat adrenal gland; 2 different PPTs	2 ISs	Gradient; 15 min /10 min; 2	R: 85-110; ME: NS, except DOPAC	(Gu et al., 2008)

Table 5. Overview of methods applied for determination of neurotransmitters and its metabolites in tissue and cells in the literature. Abbreviations: AD: adrenaline; ADP: adenosine 5'-diphosphate; AMP: adenosine 5'-monophosphate; BCCs: bovine chromaffin cells; DA: dopamine; DOPAC: 3,4-Dihydroxyphenylacetic acid, metabolite of DA; GABA: γ-aminobutyric acid; Glu: glutamic acid; MHPG: 3-Methoxy-4 hydroxyphenylglycol, metabolite of NA and AD; MN: metanephrine, metabolite of AD; NA: noradrenaline; 5-HIAA: 5-hydroxyindoleacetic acid, metabolite of 5-HT; 5-HT: serotonin; HIST: histamine; HPLC-ED: high performance liquid chromatography-electrochemical detection: HPLC-FLD: high performance liquid chromatography-fluorescence detection; HPLC-UV, high performance liquid chromatography-ultraviolet detection; INY: injection volume; IS: internal standard; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LLE: liquid-liquid extraction; LLOQ: lower limit of quantification; ME: matrix effect; MENK: methionine-enkephalin; NI: not informed; NTT: no treatment; NS: not significant; PPT: protein precipitation; R: extraction recovery; RIA: radioimmunoassay; SPE: solid phase extraction.

Method	Analyte(s)	Sample type; extraction procedure	IS	Separation type; time; INY (µL)	Recovery; Matrix effect (%)	Author
LC-MS/MS	GABA, Glu	Rat brain microdialysis; NI	Isotope labeled ISs	Gradient; 3 min; 10	NI	(Buck et al., 2009)
	2 methods: 5-HT, NA, DA, 5-HIAA, MHPG (+2 more)	Rat brain tissue; LLE	NI	Isocratic; 7 min/10 min; 5/20	R: 70-108; ME: NI	(Su et al., 2009)
	NA, 5-HT, MENK, LENK	Rat brain tissue; SPE/LLE	2 ISs	Gradient; 17 min; 20	R: 70-79; ME: NS	(Xu et al., 2011)
	NA, AD, DA, 5-HT, GABA, Glu	Rat brain tissue; PPT	Isotope labeled	Gradient; 10 min; 5	R: 81-106; ME: 50-78	(Zhu et al., 2011)
UPLC-MS/MS	GABA, Glu, DA, 5-HT, 5-HIAA (+4 more)	Rat brain tissue; PPT	NI	Gradient; <8 min; 5	R: 68-112; ME: NS	(Gonzalez et al., 2011)
	5-HT, 5-HIAA, DA, NA, GABA, Glu (+2 more)	Rat brain tissue; PPT	1 IS	Gradient; <9 min; 5	R: 84-105; ME: 64-108	(He et al., 2013)
LC-MS/MS	**NA, AD, DA, GABA, Glu, 5-HT, 5-HIAA, MHPG**	**Rat brain tissue; PPT**	**1 IS**	**Gradient; 14 min; 5**	**R: 87-114; ME: 63-106**	***(Wojnicz et al., 2016b)**
	NA, AD, MN, DA, GABA, Glu, 5-HT, 5-HIAA, HIST ADP, AMP, cAMP, MENK, LENK	**BCCs secretion and content; NTT**	**2 ISs, one isotope labeled**	**Gradient; 16 min; 5**	**R: NI; ME: 92-118 NS, except Glu**	***(Wojnicz et al., 2016a)**
	NA, 5-HT (+9 more)	Mouse/non human primates plasma, CSF, brain tissue; PPT	Isotope labeled	Gradient; 5.5 min; 20	R: NI; ME: NS	(Fuertig et al., 2016)
UPLC-MS/MS	DA, NA, AD (+3 more)	Rat brain microdialysates; micro-LLE	1 IS	Gradient; 4.5 min; 2	R: 95-106; ME: 97-109	(He et al., 2016)
	DA, 5-HT, 5-HIAA, NA, Glu, GABA (+3 more)	Rat/mouse brain and ECF; rapid PPT	Isotope labeled ISs	Gradient; 5.2 min; 7-7.5	R: 100-101; ME: 89-137	(Bergh et al., 2016)
LC-HRMS	NA, AD, DA, GABA, 5-HT, 5-HIAA, MHPG (+3 more)	Mouse hypothalamus tissue, PPT	2 ISs	Gradient; 18 min; 10	R: 82-118; ME: NI	(Yang et al., 2016)
DESI-IMS	DA, 5-HT, Glu, GABA (+5 more)	Native rat brain tissue; NI	Isotope labeled ISs	NA	NI	(Shariatgorji et al., 2016)

Table 5 continuation. Overview of methods applied for determination of neurotransmitters and its metabolites in tissue and cells in the literature. Abbreviations: AD: adrenaline; ADP: adenosine 5'-diphosphate; AMP: adenosine 5'-monophosphate; BCCs: bovine chromaffin cells; DA: dopamine; DESI-IMS: desorption electrospray ionization-imaging mass spectrometry; GABA: γ-aminobutyric acid; Glu: glutamic acid; MHPG: 3-Methoxy-4 hydroxyphenylglycol, metabolite of NA and AD; MN: metanephrine, metabolite of AD; NA: noradrenaline; 5-HIAA: 5-hydroxyindoleacetic acid, metabolite of 5-HT; 5-HT: serotonin; HIST: histamine; HPLC-ED: high performance liquid chromatography-electrochemical detection: HPLC-FLD: high performance liquid chromatography-fluorescence detection; HPLC-UV, high performance liquid chromatography-ultraviolet detection; INY: injection volume; IS: internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLE: liquid-liquid extraction; LLOQ: lower limit of quantification; LC-HRMS: liquid chromatography-high resolution mass spectrometry; ME: matrix effect; MENK: methionine-enkephalin; NI: not informed; NTT: no treatment; NS: not significant; PPT: protein precipitation; R: extraction recovery; RIA: radioimmunoassay; SPE: solid phase extraction; UPLC-MS/MS: ultra high performance liquid chromatography-tandem mass spectrometry. *The results obtained with the method we developed are highlighted in green.

2.1. Method validation (Articles 3 and 4)

First, an analytical method for 8 NTs and their metabolites in rat brain (Wojnicz et al., 2016b) was developed and validated. Then, applying some modifications and improvements to the previous method allowed us to simultaneously quantify 14 NTs, this time in BCCs (Wojnicz et al., 2016a).

MS/MS conditions

ESI was selected for both of our analytical methods: 8 NTs and their metabolites in rat brain (Wojnicz et al., 2016b), and 14 NTs in BCCs (Wojnicz et al., 2016a). Concerning the first method, ESI in positive mode was selected for 6 NTs and 5-HIAA, and ESI in negative mode was applied for MHPG. ESI in positive mode was chosen for all compounds in the case of the second method.

MRM was chosen for *m/z* recording for both methods; however, there were some differences in the MRM scan mode applied. Segment MRM method was applied in the first method (Wojnicz et al., 2016b). The MRM method was performed in 3-time segments depending on the RT of each compound. Owing to the different polarity applied for 6 NTs and 5-HIAA (positive), and for MHPG (negative), it was the only way to prolong the scan time on selected *m/z* in each segment and to improve the resolution of each analyte. Concerning the second validated method, dynamic MRM (dMRM) was performed for 14 NTs in BBCs (Wojnicz et al., 2016a). To optimize dMRM method, it was necessary to determine the RT for each compound separately and then to define an RT window. This way the method development takes more time, however it has an important advantage as it prolongs the scan time for each compound in a previously defined RT window. Therefore, the instrument only performs scanning of selected *m/z* around the defined elution time (RT window) of each compound instead of recording continuously throughout the whole chromatogram. Thus, dMRM scan mode significantly improves peak shape and selectivity of the analytes. However, only analytes with the same polarity can be included in such a method.

Thanks to the segment MRM and dMRM, we could include all 8 NTs and their metabolites in rat brain (Wojnicz et al., 2016b) and all 14 NTs in BCCs (Wojnicz et al., 2016a), respectively, in only 1 chromatographic run, while other authors had to apply 2 different methods: one for compounds with negative polarity (mostly NTs metabolites) and another one for those with positive polarity (Chritton et al., 1997; Gu et al., 2008; Su et al., 2009). Moreover, that kind of solution often required 2 different extraction

methods and 2 different mobile phases applied in chromatographic run, which obviously prolongs the total time of analysis.

Calibration curve, selectivity and sensitivity

The concentration range data, including LLOQs achieved by each method, are difficult to compare between publications due to the different units used. Although we performed different adjustments for different analytes and different concentration ranges of calibration curves (linear, polynomial and power regression models), we obtained satisfactory correlation coefficient (R^2) for both method, similar to other methods (Carrera et al., 2007; Gonzalez et al., 2011; Gu et al., 2008; Su et al., 2009).

The present approaches were selective, as no interferences were found in the detection of the analytes. Of note, ion source and gradient modifications have improved catecholamines response in our 2nd method (Wojnicz et al., 2016a) compared to our first method (Wojnicz et al., 2016b). However, the presence of salts from Krebs Hepes buffer (KH) in the BCC samples made it impossible to obtain lower LLOQs in real samples. Nevertheless, LLOQs were set correctly for all NTs found in the rat brain tissue and mouse hippocampus (1st method), basal secretion and intracellular content in BCC samples (2nd method). 5-HIAA, ADP and cAMP presented a higher LLOQ than that required for BCC secretion samples quantification (2nd method). Taking into account that mainly nucleotides have been affected, it may be explained by the chemical properties and the pH of 2.1 causing the poor retention of the nucleotides in the column.

In detail, LLOQs achieved for 8 NTs and their metabolites in rat brain tissue were similar to those reported by Gonzalez and co-workers (Gonzalez et al., 2011). While the Zheng group reported better LLOQs for NA and 5-HT brain monitoring (Zheng et al., 2012), other authors were not able to achieve such good results (He et al., 2013; Su et al., 2009; Xu et al., 2011). It can be explained by MS/MS scan mode, simple MRM provides less sensitivity than segment MRM used in our method. Similarly, segment MRM used in our method allowed us to achieve the most sensitive LLOQ (0.25 ng/mL) to detect AD in rat brain samples. Nguyen and co-workers have also included AD in their method (Nguyen et al., 2010), but they did not use segment MRM, which could be a possible reason to obtain less sensitive LLOQ than ours (1 ng/mL). Finally, the LLOQs obtained with our 2nd method were lower than those obtained in BCC cultures by Carrera and co-workers (Carrera et al., 2007) for NA, AD and 5-HT, and the LOD was also lower for DA. In addition, we obtained at least 10

times lower LLOQs for AD, NA, DA and MN than those determined in rat adrenal gland (Gu et al., 2008). This could be related to the use of dMRM mode in our method, leading to prolonged scan times for each *m/z* and thus improving the method's sensitivity.

Sample extraction procedure, recoveries and matrix effect

The method chosen for the sample preparation will influence recoveries and matrix effect of the analytes. Thus, the extraction method should be well optimized to guarantee results acceptable by EMA and FDA regulatory agencies. Many authors have used derivatization process in sample preparation (Davis et al., 1978; He et al., 2013; Wu et al., 2013; Yoshitake et al., 2003). However, this process is time consuming and it often negatively influences analyte selectivity (Zhang et al., 2007), thus it is not recommended for LC-MS/MS analysis. Therefore, to extract 8 NTs and their metabolites from rat brain (Wojnicz et al., 2016b), we have applied a simple tissue homogenization with 1.89% formic acid, followed by PPT using ACN with 1% formic acid. The mean percentage of recoveries obtained with this extraction method were between 87-114%; relative values were calculated with IS, isoprenaline. Other authors used PPT as extraction method and non isotope-labeled ISs, reporting recoveries ranging from poor (70-79% (Xu et al., 2011)) to very high percentages near 100% (Nguyen et al., 2010). It seems some of the best results were obtained using isotope-labeled ISs, as in the case of Bergh and co-workers (100-101% (Bergh et al., 2016)), Wu and co-workers (95-105% (Wu et al., 2013)) or the Zhu's group (81-106% (Zhu et al., 2011)). However, isotope-labeled ISs do not always guarantee the best result (Xu et al., 2011). The matrix effect achieved in our method was between 80-118% (except for Glu with a value of 63%). Actually, one of the limitations of our study is the lack of isotope-labeled IS for each compound. For this reason our results are acceptable, but better results using of isotope-labeled IS can be found in the literature. The slight ion suppression observed for Glu, despite the use of non isotope-labeled IS, can be explained by a high endogenous concentration of this compound in the rat brain matrix. Low Glu matrix effect values of 50% or 60% were also reported by other authors (He et al., 2013; Zhu et al., 2011).

To determine 14 NTs in BCC samples (Wojnicz et al., 2016a), we performed 2 centrifugation steps and dilution and used only 2 ISs: D Ala^2-LENK (isotope-labeled) and isoprenaline (non isotope-labeled). Mobile phase was used for sample dilution to decrease salt content (caused by KH, which is not compatible with LC-MS/MS ESI analysis). Other authors have reported similar preparation for similar sample types and

LC-MS/MS detection (Carrera et al., 2007; Qian et al., 2004). Consequently, sample preparation was fast and simple. However, salts remain in the sample and may interfere with ESI source and cause imprecisions in LC-MS/MS analysis (Vogeser and Seger, 2010). Actually, this is another limitation of our study. Future experiments with isotope-labeled IS for each compound and SPE sample preparation procedure will be required to improve the results. This improvement is needed, especially when applying this method for other complex matrices, such as human plasma.

Chromatographic separation, time of analysis and injection volume

When compounds are weakly retained and represent similar physical-chemical properties, a gradient HPLC is recommended for their good separation (Schellinger and Carr, 2006). This is exactly the case for some NTs and their metabolites. For this reason, most of the described methods (Table 5) have chosen gradient separation (Bergh et al., 2016; Carrera et al., 2007; Chritton et al., 1997; Davis et al., 1978; Gonzalez et al., 2011; Gu et al., 2008; He et al., 2013; He et al., 2016; Murai et al., 1988; Wojnicz et al., 2016a; Wojnicz et al., 2016b; Xu et al., 2011; Zhu et al., 2011). Although methods with isocratic chromatographic separation were also described (Muller and Unsicker, 1981; Wu et al., 2013; Yoshitake et al., 2003), most of them include only a few compounds in the analytical run and the time of analysis is typically longer compared to the gradient separation.

The conditions of mobile phase were first determined by our group for 8 NTs and their metabolites in rat brain (Wojnicz et al., 2016b) and then with some modifications applied to detect 14 NTs in BCC samples (Wojnicz et al., 2016a). Both methods employed gradient conditions for analyte separation, obtaining a single sample run time of 10 min (1st method, (Wojnicz et al., 2016b) and 12 min (2nd method, (Wojnicz et al., 2016a), followed by 4 min post-run to reequilibrate the column. In this regards, time of chromatographic separation should be as short as possible, because some compounds are unstable due to their high tendency to oxidate (catecholamines AD, NA, DA (Bicker et al., 2013)), or to lose phosphate group via enzymatic dephosphorylation (ADP). Of note, the majority of published methods for NTs and/or their metabolites determination in brain tissue require a longer time of analysis (25-42 min (Chritton et al., 1997; Davis et al., 1978; Nguyen et al., 2010; Parrot et al., 2011; Yoshitake et al., 2003)). However, some methods with shorter times of analysis (4-9 min) have also been reported (Bergh et al., 2016; Gonzalez et al., 2011; He et al., 2013; He et al., 2016). The latter have applied UPLC, which offers shorter analyte separation time due to a higher pressure in the UPLC system.

The pH of the mobile phase for both methods developed in our laboratory was set at 2.1. For our 1st method (Wojnicz et al., 2016b), the mobile phase pH optimization was slightly easier, because of the presence of 2 different groups of analytes: monoamine NTs, their metabolites and amino acid NTs. These compounds are normally stable and achieve a good chromatographic separation at acidic pH within the range of 2 to 4. Some authors have set their mobile phases at pH ranges from 2 to 4 (Carrera et al., 2007; Garcia et al., 1994; Gu et al., 2008; Muller and Unsicker, 1981), others only give the information concerning formic or acetic acid/ammonium formiate or acetate acidified mobile phase (Buck et al., 2009; Gonzalez et al., 2011; He et al., 2013; Lang et al., 2004; Su et al., 2009). For our 2nd method (Wojnicz et al., 2016a) development, where 4 different group of chemical compounds were included (monoamine and their metabolites, amino acids, neuropeptides and nucleotides), we have tested different pH's of mobile phase to optimize an acceptable chromatographic separation. For example, optimum pH for nucleotides AMP and ADP separation reported in the literature is 7 (Qian et al., 2004; Ushimaru and Fukushima, 2003). The pH inside chromaffin cell vesicles in rat is 5.5 (Winkler and Westhead, 1980). However, pH higher than 4 was not recommended for our method, given the presence of catecholamines, which are quickly degradated at neutral or high pH. Finally, after different pH's testing (from 2.1, 3.5, 4.0, 4.6 to 5.5), we decided to set it at 2.1 as it allowed for a good peak resolution and an acceptable peak area.

Extremely polar compounds with low molecular weight are difficult to detect with conventional LC-MS/MS. To solve this problem, many authors have applied ion-pairing reagents to increase RT of those compounds on C18 column (Kotake et al., 1985; Nguyen et al., 2010; Qian et al., 2004; Tareke et al., 2007). Ion-pairing reagents are not recommended for LC-MS/MS analysis with ESI due to their high tendency to produce ion suppression and MS source contamination. To avoid ion-reagents, the use of HILIC system can be applied (Buck et al., 2009). However, HILIC is limited only for certain NTs groups and the cost is high. We have used C18 column with pentafluorophenyl group for both methods. This column is recommended for polar compounds separation (similar to HILIC), but it has a better resolution and improves analyte separation compared to ordinary C18 column due to π-π interactions with the aromatic ring and NH_2 and OH groups.

Injection volume is other aspect to discuss in the analytical method development. Sample injection volume of 10-20 µL (Del Pino et al., 2011; Wu et al., 2013; Yoshitake et al., 2003) is perfectly acceptable for HPLC-ED or HPLC-FLD.

Although Parrot and co-workers were able to inject only 1 µL of sample, they have used UPLC-ED instrument. Another example of a very small volume of sample injection (1-2 µL) can be found in the method developed by He and co-workers (He et al., 2016), but is again caused by the use of UPLC-MS/MS instrument. Large volume of injected sample is not recommended when LC-MS/MS with ESI is used. Actually, sample injection volume greater than 5 µL is one of the main factors responsible for matrix effect, especially with high concentration of endogenous compounds and complex matrices. However, methods with large sample volume between 20-40 µL injected to the LC-MS/MS system (Carrera et al., 2007; Xu et al., 2011; Zhang et al., 2012) still exist. For both methods developed in our laboratory (Wojnicz et al., 2016a; Wojnicz et al., 2016b), we have injected 5 µL of sample to the LC-MS/MS instrument, similar to other authors (Gonzalez et al., 2011; He et al., 2013; Zhu et al., 2011). During the method optimization, we have tried to reduce injection volume to 2.5 µL; however, the peak area of some analytes (MENK, LENK) was too low to be acceptable for the method validation.

Carry-over

Unfortunately, many authors do not take into account the carry-over effect during the method development and validation. In my opinion, carry-over evaluation is very useful, when a method is being developed. It provides important information about HPLC injector needle and analytical column contamination. It should be especially evaluated when a high concentration of endogenous compounds (for example GABA and Glu in rat brain) are present in the sample, as they can cause serious system contamination. In both of our methods, we have evaluated carry-over effect. In the method development, due to the high endogenous concentration of GABA and Glu in rat brain (Wojnicz et al., 2016b) and of AD and NA in BCC samples (Wojnicz et al., 2016a), we had to include more needle wash injections and we also modified column wash program, to decrease carry-over phenomenon and ensure good precision and accuracy of the assay. Thus, no significant carry-over effect was observed in neither of the designed methods, according to the EMA (EMA, 2011) and FDA (FDA, 2001) recommendations. Among the 31 analytical methods included in the **table 5**, only ours and one another method developed by Bergh and co-workers (Bergh et al., 2016) have studied carry-over effect, highlighting the lack of carry-over testing concerning the so-far available methods.

2.2. Application of neurotransmitter methods

All of the discussed methods have been successfully applied for the quantification of NTs and/or its metabolites. Our method of 8 NTs and their metabolites in rat brain (Wojnicz et al., 2016b) has been successfully applied to study on one hand healthy rat brain and on the other hand hippocampus of the Nrf2 knockout (KO) murine depression model. The method assessing 14 NTs (Wojnicz et al., 2016a) has been applied to assay intracellular content and secretion profile of BCCs. Subsequently, I will discuss each of these applications separately.

2.2.1. Application 1: healthy rat brain (Article 3)

Applying the 1st method (Wojnicz et al., 2016b), we were able to determine all 8 compounds: AD, NA, DA, 5-HT, Glu, GABA, MHPG and 5-HIAA in adult male Sprague-Dawley rat brain samples without hippocampus. **Table 6** shows mean concentrations of the studied NTs and their metabolites in 7 rat brain samples determined with our method (Wojnicz et al., 2016b) and compared to the literature (see page 210). The results obtained with our method are highlighted in green.

NTs levels, such as NA, DA, 5-HT, were similar to those described by other authors (Del Pino et al., 2011; Gonzalez et al., 2011; He et al., 2013; Zhu et al., 2011). The endogenous concentration of monoamine NTs was significantly lower (about 1000 times) than that of the endogenous levels of GABA and Glu. These findings mimic the situation in human brain, where Glu followed by GABA amino acid are the most predominant neurotransmitters (Meldrum, 2000). It is noticeable that comparing 6 described methods in the bibliography, only our method was sufficiently sensitive to determine AD levels in the rat brain. The endogenous levels of MHPG were low, but similar to the results reported by Del Pino and co-workers (Del Pino et al., 2011), while lower than those described by Su and co-workers (Su et al., 2009). It can be explained by the use of positive ESI for MHPG detection in our case; while Su and colleagues used a more appropiate method for monoamines metabolites - negative ESI. Similarly, our results concerning 5-HIAA were much lower than those described by other groups included in the **Table 6** (Del Pino et al., 2011; Gonzalez et al., 2011; He et al., 2013; Su et al., 2009). This could be related to the differences in the sample storage conditions before the extraction and the analysis. The endogenous concentration of Glu was high in some methods included in the table (Gonzalez et al., 2011; Zhu et al., 2011); however levels measured by our method were the highest. Finally, GABA brain levels show a high variability between reported assays, due to differences in sample

preparation techniques and different HPLC methods applied. Nevertheless, our results are quite similar to those obtained by Zhu and co-workers (Zhu et al., 2011).

It is known that monoamines (AD, NA, DA and 5-HT) and amino acid (GABA and Glu) NTs play an important role in neurodegenerative diseases, such as Parkinson's disease, schizophrenia (Stahl, 2008) and Alzheimer's disease (Advokat and Pellegrin, 1992). Therefore, their determination within the brain research is one of the most interesting topics of investigation. Simultaneous determination of NTs and their metabolites gives the information about their concentration in a real sample. However, when each brain structure is analysed separately, it gives better information about NTs localization in brain (Del Pino et al., 2011). Consequently, the disadvantage of the majority of the analytical methods discussed herein is that the brain tissue samples are homogenized as a whole, and processed before injecting into the LC-MS/MS system. Meanwhile, new approaches using IMS techniques are emerging (e.g. DESI-IMS method described by Shariagorji (Shariatgorji et al., 2016)) that permit to monitor NTs directly in native rat brain tissue allowing to study NTs spatial distribution *in situ*.

Compound	Concentration (n=7) Mean +/- SD	Author
AD (ng/g) tissue	NI	(Su et al., 2009; Zhu et al., 2011; [1]Del Pino et al., 2011; [2]Gonzales et al., 2011; He et al., 2013)
	14.30 +/- 1.07	***(Wojnicz et al., 2016b)**
NA (ng/g) tissue	1775.32 +/- 548.39	(Su et al., 2009)
	290.00 +/- 34.00	(Zhu et al., 2011)
	413.43 +/- 8.74	[1](Del Pino et al., 2011)
	NI	[2](Gonzales et al., 2011)
	452.10 +/- 23.500	(He et al., 2013)
	356.19 +/- 65.24	***(Wojnicz et al., 2016b)**
MHPG (ng/g) tissue	194.68 +/- 28.64	(Su et al., 2009)
	NI	(Zhu et al., 2011; [2]Gonzales et al., 2011; He et al., 2013)
	43.06 +/- 2.18	[1](Del Pino et al., 2011)
	89.03 +/- 48.29	***(Wojnicz et al., 2016b)**
5-HIAA (ng/g) tissue	779.24 +/- 138.11	(Su et al., 2009)
	NI	(Zhu et al., 2011)
	748.34 +/- 16.72	[1](Del Pino et al., 2011)
	500.00 +/- 230.00	[2](Gonzales et al., 2011)
	368.20 +/- 40.00	(He et al., 2013)
	27.87 +/- 14.20	***(Wojnicz et al., 2016b)**
DA (ng/g) tissue	650.00 +/- 110.00	(Su et al., 2009)
	760.00 +/- 20.00	(Zhu et al., 2011)
	1493.04 +/- 34.28	[1](Del Pino et al., 2011)
	2200.00 +/- 980.0	[2](Gonzales et al., 2011)
	410.00 +/- 80.00	(He et al., 2013)
	1470.00 +/- 250.00	***(Wojnicz et al., 2016b)**
5-HT (ng/g) tissue	890.00 +/- 360.00	(Su et al., 2009)
	490.00 +/- 90.00	(Zhu et al., 2011)
	1232.95 +/- 24.03	[1](Del Pino et al., 2011)
	1050.00 +/- 600.00	[2](Gonzales et al., 2011)
	1250.00 +/- 230.00	(He et al., 2013)
	1550.00 +/- 170.00	***(Wojnicz et al., 2016b)**
Glu (µg/g) tissue	NI	(Su et al., 2009; [1]Del Pino et al., 2011)
	1408.92 +/- 141.83	(Zhu et al., 2011)
	1246.40 +/- 139.90	[2](Gonzales et al., 2011)
	835.00 +/- 54.00	(He et al., 2013)
	8439.19 +/- 1435.29	***(Wojnicz et al., 2016b)**
GABA (µg/g) tissue	NI	(Su et al., 2009; [1]Del Pino et al., 2011)
	445.15 +/- 110.77	(Zhu et al., 2011)
	51.95 +/- 8.75	[2](Gonzales et al., 2011)
	2453.00 +/- 341.00	(He et al., 2013)
	291.91 +/- 60.92	***(Wojnicz et al., 2016b)**

Table 6. Mean concentration of neurotransmitters and their metabolites of 7 rat brain samples, measured by the method we developed, in comparison with the literature. Modified from Wojnicz et al., 2016b. The results are listed in order of the year of publication, from less to more recent. Data are expressed as mean +/- SD of the total number of experiments (n= 7) in ng/g or µg/g of brain tissue for each analyte. Abbreviations: AD: adrenaline; DA: dopamine; GABA: γ-aminobutyric acid; Glu: glutamic acid; MHPG: 3-Methoxy-4 hydroxyphenylglycol ; NA: noradrenaline; 5-HIAA: 5-hydroxyindoleacetic acid; 5-HT: serotonin; SD: standard deviation; NI: not informed
[1](Del Pino et al., 2011): the main concentration value was calculated from 8 rat brain structures: hypothalamus, midbrain, cerebellum, medulla oblongata, brainstem; prefrontal cortex, striatum and hippocampus (n=12); [2](Gonzalez et al., 2011): the main concentration value was calculated from 4 rat brain structures: prefrontal cortex, striatum, nucleus accumbers and amygdala (n=4) . *The results obtained with the method we developed are highlighted in green.

2.2.2. Application 2: murine depression model (Article 3)

The same method for 8 NTs and their metabolites in rat brain (Wojnicz et al., 2016b) was also successfully applied to the murine Nrf2 model of depression. Hippocampus samples of Nrf2 KO mice (Nrf2 (−/−)) were used as a model of depression and compared with Nrf2 wild-type (WT) (+/+) mice. **Table 7** shows mean concentrations of measured NTs and their metabolites (NA, 5-HT, DA, 5-HIAA, GABA and Glu) in Nrf2 KO (−/−) and WT (+/+) mice. GABA levels decreased significantly in Nrf2 (−/−) mice compared to Nrf2 (+/+) mice, while other NTs did not show statistical differences. These results are similar to those found by Martín-de-Saavedra and coworkers (Martin-de-Saavedra et al., 2013), also reporting a lack of statistically significant differences for 5-HT, Glu levels in the hippocampus, and DA levels were under LOD. However, in a different study using extracts from the prefrontal cortex, 5-HT and DA levels were significantly reduced, while Glu levels were significantly increased (Martin-de-Saavedra et al., 2013). In this latter study, GABA levels were not determined. Therefore, ours is the first report of hippocampal decrease in GABA levels in Nrf2 (−/−) mice. Our finding supports the hypothesis that GABA deficit can cause depression (Luscher et al., 2010). In the literature, reduced GABA levels were also reported in human samples from depressive patients, such as brain (Sanacora et al., 1999), plasma (Petty and Sherman, 1984) and CSF (Gerner and Hare, 1981) samples.

	NA (ng/g tissue)	5-HT (ng/g tissue)	DA (ng/g tissue)	5-HIAA (ng/g tissue)	GABA (µg/g tissue)	Glu (µg/g tissue)
Nrf2(+/+)/vehicle	310 +/- 40	220 +/- 30	11.72 +/- 1.7	100 +/- 20	720 +/- 110	1900 +/- 200
Nrf2(-/-)/vehicle	320 +/- 40	180 +/- 40	15.18 +/- 4.0	70 +/- 10	440 +/- 60*	2300 +/- 300

Table 7. Hippocampal neurotransmitter levels measured by our method in the Nrf2 knockout mouse model of depression. Abbreviations: DA: dopamine; GABA: γ-aminobutyric acid; Glu: glutamic acid; NA: noradrenaline; Nrf2: nuclear factor (erythroid 2-derived)-like 2; 5-HT: serotonin; 5-HIAA: 5-hydroxyindoleacetic acid, metabolite of 5-HT. Data are expressed as mean +/- S.E.M. (n= 5–7). Statistical analysis was performed using an unpaired 1-tailed t-test. *$p < 0.05$ vs Nrf2. Taken and modified from Wojnicz et al., 2016b.

Furthermore, Freitas and co-workershave recently applied our methodology (Wojnicz et al., 2016b) to study the effect of agmatine on NTs levels in the corticosterone-treated mouse as a model of depression (Freitas et al., 2016). **Table 8** shows their results, which indicate that chronic agmatine treatment is able to induce significant modification/normalization of endogenous NTs levels (NA, DA, 5-HT and Glu) previously changed with depression-like behavior induced by corticosterone (Freitas et al., 2016).

	NA (ng/g tissue)	5-HT (ng/g tissue)	DA (ng/g tissue)	Glu (µg/g tissue)
Vehicle/vehicle	44.9 +/- 3.2	24.7 +/- 2.5	1.5 +/-0.1	2600 +/- 400
Agmatine/vehicle	96.5 +/- 8.8**	41.7 +/- 6.3**	14.6 +/- 3.9**	1700 +/- 200
Vehicle/CORT	41.7 +/- 4.7	11.3 +/- 0.9*	1.4 +/- 0.06	4100 +/- 300**
Agmatine/CORT	83.6 +/- 11.3##	35.8 +/- 5.5##	11.2 +/- 2.1##	2400 +/- 400##

Table 8. Effect of agmatine (0.1 mg/k/day, p.o.) in mice chronically (21 days) treated with CORT (20 mg/kg/day, p.o) on hippocampal neurotransmitters levels using liquid chromatography-tandem mass spectrometry. Abbreviations: CORT: corticosterone; DA: dopamine; Glu: glutamic acid; NA: noradrenaline; Nrf2: nuclear factor (erythroid 2-derived)-like 2; 5-HT: serotonin. Data are expressed as mean +/- S.E.M. (n=5). Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test *p<0.05 and **p<0.01 compared with the control group (vehicle/vehicle); #p<0.05 and ##p<0.01 compared with the vehicle/CORT group. Taken and modified from Freitas et al., 2016.

To summarize, our method for assessing 8 NTs and their metabolites in rat brain (Wojnicz et al., 2016b) was successfully applied for rat and mouse brain samples, and it is already being exploited by other authors. We believe that with some sample extraction modifications and improvements the method can be applied for human plasma and CSF studies, allowing the identification of possible biomarkers of depression and other neuropsychiatric disorders (e.g. neurodegenerative diseases).

2.2.3. Application 3: bovine chromaffin cells (Article 4)

The Dale's hypothesis ''one neuron, one transmitter'' was valid for decades (Dale, 1935). However, recent findings have revealed co-release of neurotransmitters by neural cells (Burnstock, 2004; Gutierrez, 2008; Hnasko and Edwards, 2012). The co-existence and co-secretion of catecholamines, neuropeptides and nucleotides from chromaffin cells have been described in the literature (Castillo et al., 1992; Crivellato et al., 2008; Podvin et al., 2015; Sillero et al., 1994; Todorov et al., 1996; Winkler, 1993). Most recently, the evidence about the co-existence and co-release of catecholamines with GABA (Harada et al., 2016; Harada et al., 2010; Inoue et al., 2010; Matsuoka et al., 2008) and Glu (Seal and Edwards, 2006) from chromaffin cells of adrenal medulla reinforces the "paraneuron" term introduced by Fujita (Fujita, 1977) and Kobayashi (Kobayashi, 1977) in 1977 for adrenal chromaffin cells. Taking all the evidence into account, we have therefore decided to apply our method for simultaneous monitoring of 4 different group of NT: 14 NTs in BCCs (Wojnicz et al., 2016a) to study basal and ACh-stimulated secretion and intracellular content of BCC cultures.

Intracellular content

Applying our LC-MS/MS method, we were able to determinate all 14 NTs from intracellular content of BCCs: AD, NA, DA, 5-HT, MN, HIST, GABA, Glu, cAMP, AMP, ADP, LENK and MENK. The comparison of all measurements is difficult, because the current study is the first one of its kind reported in the literature. However, Podvin and co-workers determined 3 catecholamines (AD, NA and DA) and 4 neuropeptides, including MENK. They found that catecholamine levels were 1000 times higher than those of neuropeptides (Podvin et al., 2015), which we could confirm with our results, even though Podvin *et al*. determined NTs in LDCVs of BCCs, while we measured ours in BCCs intracellular content.

Moreover, the concentration data are difficult to compare between publications due to different units used. Therefore, to compare the available data, we have taken only 2 catecholamines into account (AD and NA). The sum of AD and NA intracellular content is 100%. **Table 9** shows the mean percentages data of AD and NA as measured by Muller and Unsicker (Muller and Unsicker, 1981), Kuwashima and co-workers (Kuwashima et al., 2000) and our group (Wojnicz et al., 2016a). All of the measurements gave similar results: AD content between 70-75% and NA between 30-25%. These results were expected, since the AD and NA content in BCC samples was already defined by Muller and Unsicker in 1981, as shown in the table. However, Podvin and co-workers (Podvin et al., 2015) reported different percentage for AD and NA, of 57 and 43 %, respectively. This difference can be explained by the before-mentioned fact that they measured LDCVs content and not whole BCCs content. The accuracy of our measurement is further confirmed by similar findings from rat adult adrenal gland (Gu et al., 2008). Interestingly, in human foetal adrenal gland the percentage of AD and NA were the opposite (Garcia et al., 1994), then changing with the developing process.

AD Mean content (%)	NA Mean content (%)	Sample type	Author
73.5	26.5	BCCs (cultured 3 days)	(Muller and Unsicker, 1981)
35.4	64.6	Human foetal adrenal gland (13-18 weeks old)	(Garcia et al., 1994)
75.0	25.0	BCCs (cultured 4-14 days)	(Kuwashima et al., 2000)
67.0	33.0	Rat adult adrenal gland (13-18 weeks old)	(Gu et al., 2008)
56.8	43.2	LDCVs from BCCs (cultured 7 days)	(Podvin et al., 2015)
69.6	**30.4**	**BCCs (cultured 1 day)**	***(Wojnicz et al., 2016a)**

Table 9. Total content (%) of AD and NA in the method we developed compared to the literature. *Data are expressed as mean pertentage of total intracellular content (n= 6). The sum of AD and NA is the 100%. Abbreviations: AD: adrenaline; BCCs: bovine chromaffin cells; LDCVs: Large dense core vesicles; NA: noradrenaline. *The results obtained with the method we developed are highlighted in green.

Basal and ACh-stimulated secretion (stress conditions)

Finally, our method was applied to determine the neurotransmitter profile under stress conditions. BCCs were stimulated by ACh at a high concentration (100 µM). ACh is a physiological stimulant at lower concentrations, but at high concentration (≥100 µM), it is considered to cause stress. We have achieved a significant increase of secreted NTs by the treatment, except for MENK and LENK. The increment of secreted NTs profile was 92 times for GABA, 77 times for NA, 34 times for AD and AMP, 13 times for DA, 9 times for Glu, 7 times for LENK, 5 times MN and MENK, 4 times for 5-HT and only 2 times for HIST. Of note, we reported the most complex NTs profile, including monoamine, amino acids, nucleotide and neuropeptide neurotransmitters, while other authors have determined only catecholamines (AD, NA and sometimes DA). Therefore, to compare the data, only AD and NA have been taken into account. **Table 10** displays the calculated increase of AD and NA secretion under stress conditions. The results previously reported by other authors in BCCs are similar to what we found (Kuwashima et al., 2000; Teraoka et al., 1993): 1) there is an increment of catecholamine secretion under stress conditions and 2) a predominance of NA secretion over AD secretion in the bovine adrenal medulla. The reason for higher NA secretion lies in better effectiveness of secretory apparatus of NA cells in comparison to AD cells of the bovine adrenal medulla (Teraoka et al., 1993) and also in differences among mitochondrial subpopulations (Caricati-Neto et al., 2013). Of note, our method is the only one able to measure not only catecholamines, but also other neurotransmitters relevant under stress conditions (e.g. GABA and Glu, which are involved in Alzheimer's disease and depression (Kim et al., 2016)). Curiously, our results uncover a significant concentration increase of GABA and Glu under stress condition compared to basal secretion. Future experiments will be needed to

understand if this increase has an important role in physiopathogenesis of neurodegenerative diseases.

AD increment Basal/Stress	NA increment Basal/Stress	Sample	Author
5.6 ↑	10 ↑↑	ACh (100 µM) stimulated BCCs (cultured 4-14 days)	(Kuwashima et al., 2000)
34 ↑	**77 ↑↑**	**ACh (100 µM) stimulated BCCs (cultured 1 day)**	***(Wojnicz et al., 2016a)**

Table 10. AD and NA basal and ACh stimulated secretion (stress conditions) from bovine chromaffin cells in the method we developed compared to the literature. Data are expressed as mean ratio of AD and NA basal secretion vs stress stimuli (n= 6). Only AD and NA were taken into account. Abbreviations: ACh: acetylcholine; AD: adrenaline; BCCs: bovine chromaffin cells; NA: noradrenaline. *The results obtained with the method we developed are highlighted in green.

Additionally, our method is planned to be applied in preliminary assays to a mouse model of Alzheimer's disease and also to human plasma samples of migraine and Alzheimer's disease patients. However, some improvement in sample preparation has to be done. Some neurotransmitters, such as AD, NA, DA and enkephalins have very low endogenous levels in human plasma. Therefore, improving of the sample preparation procedure, using SPE and isotope-labeled ISs, will be required to obtain a good sensitivity of the method.

3. Application of IMS for human habitat studies

The 5th article of this thesis uses a novel untargeted screening, which allows to simultaneously measure as many compounds as possible from a biological sample without any bias (Patti et al., 2013). Compared to the methods discussed so far in the thesis, untargeted approaches provide a greater scope than targeted analysis, enabling the discovery of unknown compounds and their metabolites. While there are already some examples of untargeted studies in the literature (Wu and Colby, 2016); (Baker, 2011); (Nakayama et al., 2014), these studies represent certain challenges when it comes to their accurate validation strategies. Herein, we successfully applied 3D-surface-IMS for studying chemicals associated with 5 types of modern human habitats (approximately 200 locations for each habitat): 2 bicycles, a happy hour microenvironment, a car, an apartment and a water drinking fountain in a research building; and 19 human subjects associated with these environments. Thanks to a complex analysis, it was possible to assess the chemistry exchange between human and environment and *vice versa*. Interestingly, using this approach we found out that only 3 of the 5 selected habitats were fit for the study purpose. The remaining 2 (happy hour microenvironment and water drinking fountain in a research building) were not the best choice for human habitat-environment interaction studies, because none of these environments were routinely visited by any of the volunteers.

3.1. Method validation strategy (Article 5)

Sample preparation

As opposed to 2D methods described in the literature, where the MS instrument records and generates a 2D image, here, sample locations were recorded manually using photos and videos in order to construct 3D model maps with *x, y, z* coordinates using 3D tools. Only one similar study using the 3D-surface-IMS method exists (Bouslimani et al., 2015), and it was developed in the same laboratory as the study that is a part of this thesis. The applied extraction protocol (a simple cotton swab) has been described in the previous work (Bouslimani et al., 2015), and applied since it is much shorter than those described by other authors (Bokhart and Muddiman, 2016; Lauer et al., 2016; Nazari et al., 2016). However, the comparison of the extraction method is very difficult, because the sample type and detection instrument varied between methods.

MS data acquisition, processing and normalization

In this article, the following data analysis strategy was applied: IS intensity variation less than 10%, mass error less than 10 ppm and RT within 5 s. The acceptance protocol was similar to Bouslimani and co-workers (Bouslimani et al., 2015), although some modifications were introduced. In untargeted metabolomics experiments, the number of analytes in each sample varies (untargeted screening) and therefore the design of acceptance criteria is more difficult, often specific for each project. Therefore the comparison with the literature is not always possible.

3.2. Application of 3D-surface-IMS method (Article 5)

The relationships of the metabolomics data from the human habitat and the volunteers

The overall relationship between the metabolomes from the human habitat and the male/female volunteers revealed by the principal coordinates analysis (PCoA) plots show significant and expected separation between human and environment samples. However, there is no relationship between male or female subjects. Surprisingly, several sample types from the human habitat, such as the guitar, the bicycle handlebar, foosball table handles, and parts of a pool table, were more similar to human samples. It could be explained by the fact that many of these surfaces have received significant contact with human skin. This demonstrates that humans have a major impact on the chemistry of the surfaces they touch. When comparing only the 5 human habitats, we observed differences in the habitats' chemistry, particularly striking among the car samples. In case of only human data (19 volunteers), some chemical signature specific for each individual person was observed in the data obtained in this study.

The molecular network characteristics of the study

The data (49,669 merged spectra-nodes) indicates that for these environments and experimental conditions, the diversity of MS/MS spectra has reached saturation. Molecular families contain MS/MS spectra of analogs of molecules with nearly identical structure (e.g. that differ in a methyl group, oxygen or a double bond), spectra of molecules with similar substructures (e.g. glycosylations or fatty acid tails), but also spectra composed of different adducts but showing similar fragmentation and often source fragment ions are also detected within the molecular family. **Figure 16** depicts the percentages of the same MS/MS spectra found in each habitat compared to the

volunteers (human habitat) and common spectra between both datasets. The lowest percentage of shared spectra (~10%) was found between the water fountain and the volunteers. The largest common MS/MS spectra (~75%) between people and habitat were found in the social gathering environment. These results were expectable, since the water fountain from research building is not routinely visited by the study volunteers, thus the percentage of shared MS/MS spectra was low; while in happy hour bar habitat, where individuals are known to socialize and consume alcohol, higher human-environment interactions were observed, confirming once more the transfer of compounds.

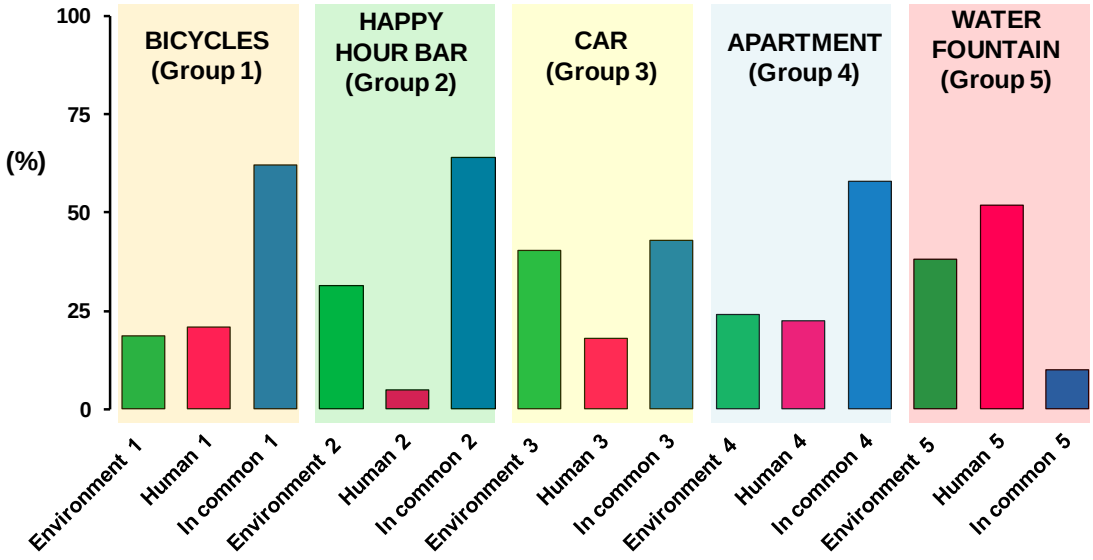


Figure 16. The percentage for the presence of the same MS/MS spectra of each habitat or mode of transportation (here labeled as environment) that was investigated compared to the volunteers (labeled as human) and the spectra that were in common between the 2 data sets. Five different groups were investigated (bicycles, happy hour bar, car, apartment and drinking water fountain). The sum of the percentages of environment, human and common (human+environment) in each of 5 groups is understood as 100%. Abbreviation: MS/MS: tandem mass spectrometry.

The annotated molecules found in this work were chemicals from:

- personal care products, e.g. CAPB, ammonium glycyrrhizinate, sclareolide, dexpanthenol
- human food, e.g. citrus flavonoids annotated as hesperidin, tangeritin, poncirin, nobilitin and isoquercetin; chocolate component epicatechin; catechin, caffeine, astragalin, theobromine or theophylline found in coffee and tea
- food additives, e.g. aspartame, sucralose and food preservatives, e.g. imazalil

- medications,e.g. the antifungals terabinafine, the antibacterial erythromycin and the anthelmintic niclocide
- derived from cleaning supplies, e.g. benzalkonium chloride
- plasticizers, e.g. phthalates
- molecules of humans and other organisms, e.g. glycocholic acid - a bile acid, mono and di acyl-glycerols, small peptides, amino acids, and other lipids, such as hexadecenoic acid methyl ester and palmitelaidic acid, the most common fatty acid in human skin
- other molecules, such as methylene blue, used to stain protein gels and ketamine, used for sedation of animals.

Of note, CAPB was detected in almost every sample studied. It was to be expected, since CAPB is a surfactant very commonly used in cleaning, detergent and personal care products and it actually often causes skin allergy (Suuronen et al., 2012). Interestingly, caffeine was detected in ≈60 samples. Taking into account that nowadays coffee, tea or other caffeine-based drinks consumption is very high (Bessonov and Khanferyan, 2016; Doepker et al., 2016), this result was not surprising. On the other hand, the antibiotic, erythromycin, one of the aquatic contaminants (Bound and Voulvoulis, 2005), was found in ≈20 samples. The drug cocaine was present only in 4 human habitat samples, but not on humans. Cocaine was expected to be found in much more samples, since traces of this drug are presented on money in U.S. and in Europe (Dixon et al., 2006; Jourdan et al., 2013; Wimmer and Schneider, 2010; Zuo et al., 2008) and in urban wastewater of Italy and U.S. (Castiglioni et al., 2011).

3D Cartography

Spatial distributions of the detectable chemicals using 3D cartography (3D-surface) were also analysed in order to visualize the compound transfer between individuals and habitats. To find spatial correlation between chemicals and habitat, 5 habitats and 19 volunteers were mapped. **Table 11** represents 5 most interesting molecules found in each human habitat and the origin of the annotated molecules. Each from 5 analysed environments had a characteristic set of molecules that were not detected in other environments. Nevertheless, more than 50% of chemicals are shared between 2 or more environments.

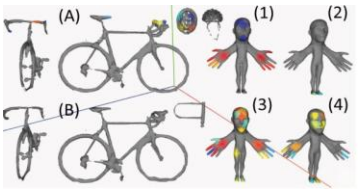
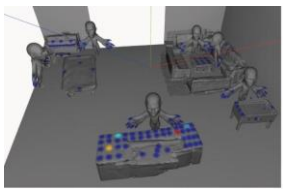
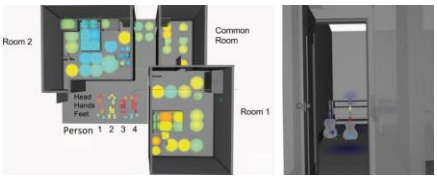
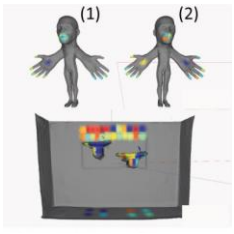
Group	Environment	Analytes	Origin
1	2 bicycles + 4 volunteers 	CAPB	**Personal care products**
		Dexpanthenol	**Personal care products**
		Ketamine	**Anesthesia and sedation**
		Caffeine	**Food**
		Aspartame	**Food**
2	happy hour room + 5 volunteers 	Dihydrocapsaicin	**Food**
		Dexpanthenol	**Personal care products**
		Procyanidin B2	**Food**
		m/z 241.141	-
		m/z 574.212	-
3	car + 4 volunteers 	Dibuthyl phthalate	**Plasticizers**
		m/z 240.232	-
		m/z 287.081	-
		m/z 501.209	-
4	apartment + 4 volunteers 	CAPB	**Personal care products**
		Terbinafine	**Medications (antifungal)**
		Cocaine	**Drug**
		m/z 495.830	-
		m/z 355.284	-
5	water drinking fountain in a research building + 2 volunteers 	CAPB	**Personal care products**
		Catechin flavonoid	**Food**
		1-Palmitoyl-sn-glycero-3-phosphocholine	**Human skin**
		Ammonium glycyrrhizinate	**Personal care products**
		Aspartame	**Food**

Table 11. Five different human habitats group selected in the present work, their environment, molecules founded in each habitat and the origin of known annotations. 3D-surface-IMS was used for the identification and visualization of chemicals. The features with *m/z* do not have an annotation, thus non origin assigned in the table. Abbreviation: CAPB: cocamidopropyl betaine; 3D-surface-IMS: three-dimensional-surface-imaging mass spectrometry; *m/z*: mass-to-charge ratio. The images taken from Petras et al., 2016.

Habitat 1: Two bicycles and 4 volunteers

In this group, CAPB was found on all volunteers and both bikes. High levels of another common cosmetics ingredient, dexpanthenol, were found on the hands of both cyclists, and in lower levels on the handlebars, the seat and the frame of one of the bicycles. These findings were likely caused by the fact that these molecules (CABP, dexpanthenol) are widely used in cosmetics. Two food originated molecules, caffeine and aspartame, were found mainly on the volunteers. Caffeine is widely consumed (Dance, 2016; Doepker et al., 2016) and was detected on 3 out of 4 individuals and also on environment: on the bike handlebars, the seat and the helmet. However, caffeine was not detected on volunteer 2. The individual 2 does not drink coffee, or tea or any caffeine containing drinks, explaining the absence of even trace amounts of caffeine. Aspartame (artificial sweetener) was identified only on volunteers, but not found on environment. High abundance of aspartame was visualized on the hands and face of volunteer 3, and at lower abundance on volunteers 1, 2 and 4. Finally, ketamine used for anesthesia and sedation, was detected primarily on volunteer 4th hands and face. Actually, this volunteer works in animal research, and had recently used ketamine for research purposes.

Habitat 2: Happy hour room and 5 volunteers

In the next habitat, where interactions between environment and individuals were more intense, we were also able to observe a transfer of the detectable chemicals from the environment to people. A food molecule, dihydrocapsaicin (an analog of capsaicin present in chili peppers) was only detected on the bar. Curry served on the bar during the social gathering or the salsa that is commonly consumed in that location are likely to be a source of dihydrocapsaicin. Procyanidin B2 (proanthocyanidin) found in apples, potatoes, including chips, was detected on both humans and the environment. These results highlight the transfer of molecules that are present in the human habitat to people. The transfer of chemicals was additionally suggested by 2 unknown compounds. Both (*m/z* 574.212 and *m/z* 241.141) showed a clear transfer between the environment and individuals interacting with it.

Habitat 3: A car and 4 volunteers

It was not surprising to find dibuthyl phthalate and other unannotated plasticizer in this environment. It is known, that this car is cleaned very thoroughly about once a month, yet many molecules are directly shared with the person that owned the car (driver seat area, floor by the gas pedals and the backseat), confirming a strong and

continuous compound transfer. Dibuthyl phthalate was detected with high intensity on the floor by the gas pedal and on shoes of the car owner. The features, not annotated in GNPS, with *m/z* 240.232 and *m/z* 287.081 were shared between individuals and the car. However, the feature with *m/z* 501.209 was detected only on individuals 2 and 3, not on the vehicle. Spatial distribution of feature with *m/z* 501.209 (face, hands, and feet) suggest a food or personal product association.

Habitat 4: A 2-bedroom apartment and 4 volunteers

As expected, a variety of lifestyle chemicals was found in this analysis. CAPB and terbinafine were shared between humans and environment. A drug, cocaine was found on electric guitar in room 2. An acoustic guitar was present in the same room, but cocaine was found only on the electric guitar purchased by second-hand. Most of cocaine was hydrolyzed indicating that the sample was old. Since one of the cocaine metabolites, benzoylecgonine, is the most stable form detected in drug tests (Castiglioni et al., 2011), it suggests the future application of this study in forense research. Feature not annotated in GNPS, with the *m/z* 495.830, was found on the shoes of every test person and on the floor of the entire apartment. Two of the people sampled do not live in the apartment, so it seems that the environment transferred it to the people. However, other not GNPS annotated compound with the *m/z* 355.284 was localized only on volunteer 1 and the room 1. It shows a specific interaction between volunteer 1 and the room 1, resulting in the transfer of chemicals between these 2 sampled surfaces.

Habitat 5: A water drinking fountain in the research building and 2 volunteers

Despite the fact that water fountain was not routinely visited by any of the volunteers, transfer of chemicals between volunteers and habitat was found. In detail, personal care-associated molecules, food derivate and human originated chemicals were found. Again, CAPB and ammonium glycyrrhizinate (a licorice derivative with a sweet taste; an additive in toothpaste and cosmetics) contributed to humans and environment habitats. Catechin flavonoid, present in polyphenol-rich food (Hahn et al., 2016), was associated with both, humans (on the lips and hands of both female and male volunteers) and the environment (the walls and water fountain). On the other hand, aspartame was only present on the male subject's tongue. This was a surprising result, because this volunteer avoids aspartame-containing food. Outstandingly, 1-Palmitoyl-sn-glycero-3-phosphocholine, which is a component of eukaryotic lipid

bilayers such as human skin, was detected with high intensity on the walls and on the water fountain, but with much lower intensity on people.

To summarize, this novel study provides insights about the transfer of chemicals between humans and modern human environment and *vice versa*. Indeed, we were able to confirm an important and continuous exchange of coumpounds and the results presented here suggest that 3D-surface-IMS together with molecular networking can be used as a chemical fingerprint of a built and transportation environment associated with the degree of human interactions. In such a way it may be possible to use such as chemical signatures of human habitats to poinout individuals who recently interacted with a specific habitat. Therefore, in the future, it can find significant application in forensic science (Lauer et al., 2016) and also solve problems in clinical medicine, the military, the criminology and astrochemistry.

CONCLUSIONS/CONCLUSIONES

As a general conclusion, we have successfully developed and validated 4 MS methods for targeted analyte screening fullfilling the requirements of regulatory agencies EMA and FDA. Moreover, we have also developed 1 untargeted metabolomics assay, which was validated according to current validation strategies. Specific conclusions of our study are listed below:

1) We successfully developed a method to determine albendazole and albendazole sulfoxide plasma levels that was applied for pharmacokinetic studies in 12 healthy volunteers. Our method uses a small volume of plasma, achieves one of the best recoveries (near 100%) and to date requires one of the shortest time of analysis.

2) We improved a mass spectrometry approach for omeprazole plasma monitoring and successfully applied it to study 240 samples of 6 healthy volunteers in order to perform bioavailability studies under fasting conditions. This method is currently the shortest assay for omeprazole plasma determination and the only one using isotope-labeled IS, which enables to achieve recovery near 100%.

3) The 3rd mass spectrometry method enabled us to simultaneously determine 8 neurotransmitters (monoamines, amino acids) and their metabolites in rat brain tissue. The method was applied in a murine depression model. Ours is the first report of reduced hippocampal GABA levels in Nrf2 mice. This finding supports the hypothesis that GABA deficit can cause depression.

4) We have successfully improved the previous method, extending its application to detect 14 neurotransmitters (monoamines, amino acids, nucleotides and neuropeptides). This is the first reported method for simultaneous determination of 14 neurotransmitters in BCCs. The assay permitted co-transmission research and with sample preparation improvements it will be applied to study plasma samples of Alzheimer's patients.

5) We have successfully performed a 3D-surface-IMS in 5 human habitats. We describe different and specific MS/MS spectra for each habitat, including distribution of chemicals, which may have an impact on the design of future human habitats.

Como conclusiones generales, hemos desarrollado y validado con éxito 4 métodos de análisis dirigido, basados en MS, según las recomendaciones de las agencias reguladoras; y un método de análisis no-dirigido, siguiendo las normas de las estrategias de validación. Las conclusiones específicas se describen a continuación:

1) Hemos desarrollado con éxito un método para la determinación simultánea del albendazol y albendazol sulfoxido en el plasma humano. El método se aplicó en los estudios farmacocinéticos de 12 voluntarios sanos. Nuestro procedimiento requiere volumenes pequeños de plasma, dando muy buenos resultados en la recuperación de la extracción (cerca de 100%) y siendo uno de los métodos más rápidos hasta ahora.

2) El método mejorado de la cuantificación del omperazol en el plasma humano se aplicó con éxito en 240 muestras humanas de 6 voluntarios sanos en un estudio de biodisponibilidad en condiciones de ayuno. El procedimiento que hemos desarollado es actualmente el más rápido y el único que usa estándares internos deuterados, garantizando buenos resultados en la recuperación de la extracción, cercana a 100%.

3) El tercer método facilita la determinación simultánea de 8 neurotransmisores (monoaminas, aminoácidos) y de sus metabolitos en el tejido del cerebro de rata. El método se aplicó con éxito en el modelo murino de depresión. Nuestros resultados son los primeros que describen los niveles del GABA reducidos en el modelo del raton Nrf2. Este hallazgo corobora la hipótesis sobre el déficit del GABA como una de las posibles causas de la depresión.

4) Se mejoró el método anterior, obteniendo 14 neurotransmisores en una sola carrera cromatográfica (monoaminas, aminoácidos, nucleótidos y neuropeptidos). Este es el primer método capaz de cuantificar simultáneamente 14 neurotransmisores en las BCCs. El procedimiento permite el estudio de neurotransmisión en este modelo celular y con la aplicación del método de extracción adecuado, se aplicará para la monitorización de los niveles endógenos de neurotransmisores en el plasma de los pacientes con la enfermedad de Alzheimer.

5) Hemos aplicado con éxito el último método de la 3D-surface-IMS para el estudio de la química asociada a 5 entornos humanos distintos. Se observó una diferente y específica distribución de las sustancias químicas en cada entorno humano, lo cual puede aplicarse en un futuro para un diseño de las viviendas y modos de transporte más consciente.

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Article 6: Imatinib assay by high-performance liquid chromatography in tandem mass spectrometry with solid-phase extraction in human plasma.

Authors: Jose María Moreno, **Aneta Wojnicz**, Juan Luis Steegman, Maria F. Cano-Abad, and Ana Ruiz-Nuño

Biomed. Chromatogr 27 (2013) 502-508

Abstract

We have developed a method of liquid chromatography in tandem with mass spectrometry to monitor therapeutic levels of imatinib in plasma, a selective inhibitor of protein tyrosine kinase. After solid-phase extraction of plasma samples, imatinib and its IS, imatinib-D8, were eluted with Zorbax SB-C18 at 60 ºC, under isocratic conditions through a mobile phase consisting of 4mM ammonium formiate, pH: 3.2 (solution A) and acetonitrile solution B. The flow rate was 0.8 mL/min with 55% solution A +45% solution B. Imatinib was detected and quantified by mass spectrometry with electrospray ionization operating in selected-reaction monitoring mode. The calibration curve was linear in the range 10-5000 ng/mL, the lower limit of quantification being 10 ng/mL. The method was validated according to the recommendations of the Food and Drug Administration, including tests of matrix effect (bias <10%) and recovery efficiency (>80 and <120%). The method is precise (coefficient of variance intra-day <2% and inter-day <7%), accurate (95-108%), sensitive and specific. It is a simple method with very fast recording time (1.2 min) that is applicable to clinical practice. This will permit improvement of the pharmacological treatment of patients.

Personal contribution: I participated in the sample processing and analysis.

Article 7: Evaluation of the Relationship between Sex, Polymorphisms in CYP2C8 and CYP2C9, and Pharmacokinetics of Angiotensin Receptor Blockers.

Authors: Teresa Cabaleiro, Manuel Román, Dolores Ochoa, María Talegón, Rocío Prieto-Pérez, **Aneta Wojnicz**, Rosario López-Rodríguez, Jesús Novalbos, and Francisco Abad-Santos

Drug Metab Dispos 41 (2013) 224-229

Abstract

Angiotensin II receptor blockers (ARBs) are used to treat hypertension. Most ARBs are metabolized by CYP2C9. The aim of this study is to evaluate the possible association between sex, polymorphisms in the CYP2C8 and CYP2C9 genes, and the pharmacokinetics of losartan, valsartan, candesartan, and telmisartan. The study population comprised 246 healthy volunteers from seven single-dose clinical trials: 64 from two candesartan studies, 43 from a telmisartan study, 36 from a losartan study, and 103 from three valsartan studies. DNA was extracted from blood samples and single-nucleotide polymorphisms in the CYP2C8 (CYP2C8*2, CYP2C8*3, CYP2C8*4, CYP2C8*5) and CYP2C9 (CYP2C9*2, CYP2C9*3) genes were evaluated using real-time polymerase chain reaction. Sex only affected telmisartan pharmacokinetics, since women showed a higher telmisartan Cmax than men (590.5 6 75.8 ng/ml versus 282.1 6 30.8 ng/ml; P £ 0.01). CYP2C9 variants were associated only with losartan pharmacokinetics: the half-life of losartan was higher in CYP2C9*3 allele carriers (3.1 6 0.4 hours) than in volunteers with the wild-type genotype (2.3 6 0.1 hours) (P £ 0.05). CYP2C8 polymorphisms were associated only with valsartan pharmacokinetics, since *2 allele carriers showed faster clearance (1.07 6 0.57 l/h·kg) than those with the wild-type genotype (0.48 6 0.72 l/h·kg; P £ 0.01) and carriers of the *3 allele (0.35 6 0.49 l/h·kg; P £ 0.001). These results suggest that genotypes for CYP2C9 and CYP2C8 are relevant to the pharmacokinetics of losartan and valsartan, respectively, but not the pharmacokinetics of candesartan or telmisartan.

Personal contribution: I participated in the sample processing.

Article 8: Agmatine, by Improving Neuroplasticity Markers and Inducing Nrf2, Prevents Corticosterone-Induced Depressive-Like Behavior in Mice.

Authors: Andiara E. Freitas, Javier Egea, Izaskun Buendia, Vanessa Gómez-Rangel, Esther Parada, Elisa Navarro, Ana Isabel Casas, **Aneta Wojnicz**, José Avendaño Ortiz, Antonio Cuadrado, Ana Ruiz-Nuño, Ana Lúcia S. Rodrigues, Manuela G. Lopez

Mol Neurobiol 53 (2016) 3030-3045

Abstract

Agmatine, an endogenous neuromodulator, is a potential candidate to constitute an adjuvant/monotherapy for the management of depression. A recent study by our group demonstrated that agmatine induces Nrf2 and protects against corticosterone effects in a hippocampal neuronal cell line. The present study is an extension of this previous study by assessing the antidepressant-like effect of agmatine in an animal model of depression induced by corticosterone in mice. Swiss mice were treated simultaneously with agmatine or imipramine at a dose of 0.1 mg/kg/day (p.o.) and corticosterone for 21 days and the daily administrations of experimental drugs were given immediately prior to corticosterone (20 mg/kg/day, p.o.) administrations. Wild-type C57BL/6 mice (Nrf2 (+/+)) and Nrf2 KO (Nrf2 (−/−)) were treated during 21 days with agmatine (0.1 mg/kg/day, p.o.) or vehicle. Twenty-four hours after the last treatments, the behavioral tests and biochemical assays were performed. Agmatine treatment for 21 days was able to abolish the corticosterone-induced depressive-like behavior and the alterations in the immunocontent of mature BDNF and synaptotagmin I, and in the serotonin and glutamate levels. Agmatine also abolished the corticosterone-induced changes in the morphology of astrocytes and microglia in CA1 region of hippocampus. In addition, agmatine treatment in control mice increased noradrenaline, serotonin, and dopamine levels, CREB phosphorylation, mature BDNF and synaptotagmin I immunocontents, and reduced pro-BDNF immunocontent in the hippocampus. Agmatine's ability to produce an antidepressant-like effect was abolished in Nrf2 (−/−) mice. The present results reinforce the participation of Nrf2 in the antidepressant-like effect produced by agmatine and expand literature data concerning its mechanisms of action.

Personal contribution: I participated in the sample processing and analysis. I also contributed to drafting the correspond part of the paper.

Article 9: Inclusion complex of ITH12674 with2-hydroxypropyl-β-cyclodextrin: Preparation, physicalcharacterization and pharmacological effect.

Authors: Patrycja Michalska, **Aneta Wojnicz**, Ana Ruiz-Nuño, Sheila Abril, Izaskun Buendia, Rafael León

Carbohydrate Polymers 157 (2017) 94-104

Abstract

ITH12674 is a multitarget drug, designed to exert a dual “drug-prodrug” mechanism of action, able to induce the phase II antioxidant and anti-inflammatory response for the treatment of brain ischemia. However, its physicochemical properties limit its potential preclinical development due to its low watersolubility and instability towards heat and pH variations. In order to improve its properties, we prepared the inclusion complex of ITH12674 with 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) by the freeze-drying method. The formation of the inclusion complex was confirmed by FT-IR spectroscopy, PXRD, DSC, 1H NMR and SEM techniques. Experimental results showed that the inclusion complex enhanced its water solubility and stability against heat, acidic and basic conditions. Furthermore, the inclusion complex, prepared in water solution, exerted the same potency to induce the phase II antioxidant response as the pure ITH12674. Thus the formation of the inclusion complex with HP-β-CD is a very effective method to stabilize and solubilize the active compound for its future preclinical development.

Personal contribution: I participated in the sample processing and analysis. I also contributed to drafting the correspond part of the paper.